ARTIFICIAL STIMULATION OF CEPHALIC CHOLINERGIC SENSORY NEURONS INDUCES MATING-LIKE MOTOR RESPONSES IN MALE *Caenorhabditis elegans*

A Thesis

by

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ABSTRACT

All complex organisms possess a nervous system which they use to monitor environmental and internal stimuli. In higher vertebrates, the nervous system is comprised of billions of cells which form highly plastic neural networks from their synapses. These large neural circuits modulate complex behaviors. The nematode roundworm *Caenorhabditis elegans* uses a small but highly-interconnected nervous system to carry out complex behaviors. The nervous system of *C. elegans* is a tractable model to determine the effects of changes on a nervous system at the systemic, cellular, genetic, and molecular levels.

The *C. elegans* male's nervous system detects environmental conditions, mating cues, attractants, repellents, and the location and composition of possible food sources and integrates these inputs to compute the decision of whether or not to mate. Mating behavior in the *C. elegans* male is regulated at a number of steps by cholinergic signaling from various sensory and sensory-motor neurons, but a comprehensive model of how cholinergic signaling controls this circuit has not yet been elucidated. Previous studies have thoroughly dissected the cellular structure, neural connectivity, and signaling pathways of the male's peripheral circuits located in the genital regions of the animal's tail. However, no studies have been conducted to determine what role the cephalic cholinergic neurons have in regulating mating behavior.

I hypothesized that cephalic cholinergic neurons exert regulatory control over the male-specific mating circuit. I inserted the transmembrane light-activated ion pore

Channelrhodopsin-2 fused to YFP and expressed from the *Punc-17small* promoter into these neurons and selectively stimulated them using high-intensity blue light. Stimulation induced mating-like behaviors in the male tail consistent with behaviors seen during copulation with a hermaphrodite. Using behavioral assays, I demonstrated that these behaviors were male-specific and only occurred after direct stimulation in the absence of a hermaphrodite. Incidence of mating-like behaviors increased significantly as the worm aged, and the mating circuit retained a memory of the stimulus, indicated by the latency between stimulation and onset of mating-like behaviors. Brief food deprivation, which normally downregulates excitability of the mating circuit via UNC-103 ERG-like K+ channels, caused an unexpected increase in the number of blue light-stimulated behaviors displayed. Pharmacological assays using acetylcholine (ACh) agonists showed that stimulation of the cephalic cholinergic neurons increased propensity for spicule protraction in the presence of an ACh agonist, and partially restored the decline in spicule protraction associated with temporary food deprivation.

I sought to identify the cephalic cholinergic neuron or neurons responsible for regulating mating-like behavior in the tail circuits. I looked for a reduction in mating-like behaviors after stimulation after removal of a cephalic cholinergic neuron pair via laser micro-ablation. Two cholinergic and chemosensory neuron pairs in the inner labial sensilla (IL2L/R and IL2VL/R) appear to generate and/or relay the signal that induces mating-like behaviors in the tail. I hypothesize that these neurons sense environmental cues before the male contacts a mate, and modulate lasting motivational changes within the male mating circuit.

DEDICATION

For my parents, brothers, and grandparents

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NOMENCLATURE

pJM1	a synthesized plasmid containing *Punc-17small:ChR2::YFP*
pha-1(e2123)	a temperature-sensitive lethal mutation halting embryonic pharyngeal development
lite-1(ce314)	a loss-of-function mutation that abolishes photophobic behavior
him-5(e1490)	a gain-of-function mutation that increases the rate of spontaneous male generation during hermaphroditic self-fertilization
ChR2	Channelrhodopsin-2, a light-cated cation channel
ATR	all-*trans*-retinal, the cofactor of Channelrhodopsin-2
Unc	"Uncoordinated" phenotype
rgIs12	The integrated line of *C.elegans* possessing the PJM1 construct
rgEx551	The injected line of *C. elegans* with variably-expressing PJM1
Egl	"Egg-laying"-related genes
ACh	Acetylcholine
AChR	Acetylcholine receptor
NIC	Nicotine
LEV	Levamisole
ARE	Arecoline
PCS	Post-cloacal sensilla
SPC	sensory-motor neurons innervating the copulatory spicules muscles
IL2	Three neuron pairs forming the inner labial sensilla

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CHAPTER I

INTRODUCTION

Caenorhabditis elegans as a model organism

Caenorhabditis elegans is a robust nematode found ubiquitously in rotting fruits around the globe [1]. It can survive a wide range of temperatures and physiological conditions. It is resist to starvation by forming a low-metabolism dauer larva, and its L1 larvae can even be frozen and stored for years at low temperatures, after which they can be thawed and be ready for experimentation after a few generations, when exposed to plentiful food [2,3].

The physiological properties of *C. elegans* make it ideal for use as a model organism, which was pioneered in the experiments of Sydney Brenner in the 1960's and 1970's [4]. At 20^{o}C, the animal has an approximately 3-day long life cycle from egg to sexually mature adult.

There are two sexes; a larger hermaphrodite (959 somatic cells) capable of self-fertilization, which possesses a vulva and two gonadal arms, in which both first spermatogenesis of approximately 300 sperm and then oogenesis occur, and a smaller, thinner male (1031 somatic cells), which possesses unique neural and muscular structures in the tail [5-7]. The hermaphrodite has a five pairs of autosomes and a pair of sex chromosomes (XX). The male worm possesses five pairs of autosomes but only a single hemizygous sex chromosome (XO). Males are typically rare in wild-type strains

because the hemizygous sex chromosome arises during self-fertilization in the hermaphrodite from the fusion of a gamete with a single sex chromosome and a nullo-X gamete (the result of a rare nondisjunction event during meiosis) [5]. Males form more frequently (approximately 50%) as the result of sexual reproduction; half of the male's gametes lack a sex chromosome because it is hemizygous. This gamete fuses with the haploid gamete from the hermaphrodite to generate a male [5].

The hermaphrodite's ability to mate sexually with a male or self-fertilize ensures both genetic variation and the ability to propagate new generations in the absence of a second animal, and results in cloned progeny. Hermaphrodites generate approximately 300 sperm which they can use to internally fertilize nearly all of their own oocytes [5]. They lay numerous eggs, ensuring a large number of animals ready for experimentation. Sexual mating permits recombination.

In wild-type *C. elegans,* males are produced at a roughly 1:1000 ratio to hermaphrodites. The *him-5(e1490)* allele promotes the formation of double-stranded breaks on the X-chromosome, increasing the rate of nondisjunction and the formation of XO hemizygous gametes [8,9]. Strains with the *him-5(e1490)* allele show an increase in the number of males spontaneously produced by hermaphroditic self-fertilization to roughly 30% [8].

C. elegans, like all organisms, is vulnerable to ultraviolet radiation. Wild-type worms will die in after as little as 25 minutes of exposure to direct high-intensity blue-violet illumination [10]. It possesses a gene, *lite*-1, which generates a light-detecting protein that mediates a photophobic rapid locomotive response (up to 3.5 times basal

locomotive rates) away from noxious ultraviolet and visible blue-violet radiation [10]. The *lite-1(ce314)* loss-of-function allele is a useful gene when carrying out photostimulation experiments in *C. elegans,* because it abolishes the natural tendency of the worm to flee high-intensity illumination [10].

The male and hermaphrodite differ in their gross morphology. Hermaphrodites have a centrally-located vulva connected to two gonadal arms which contain and fertilize maturing oocytes. The adult hermaphrodite is noticeably larger than the mature male worm, and has a long, tapered tail. The male worm possesses unique mating and sensory structures on its tail, including sclerotized copulatory spicules and the male-specific sex muscles that move them, a pair of sensilla anterior and posterior to the cloaca, the proctodeum, a restructured rectum that accommodates the release of sperm, and a fan-shaped ray sensilla (Figure 1). There are nine pairs of rays, each of which is innervated by a pair RnA and RnB neuron. With the exception of ray 6, these neurons are open to the external environment at the distal end of each ray. The RnA neurons in the 5^{th}, 7^{th}, and 9^{th} rays express dopamine. Additionally, a range of FMRFamide-like (Phe-Met-Arg-Phe) neuropeptide reporters, governed by the *flp-5, flp-6,* and *flp-17* promoters, are expressed in the RnB neurons of rays 1, 2, 3, 5, 6, 7, and 9. [11-14]..

The pioneering work of Sidney Brenner, J. White, E. Southgate, J.N. Thomson, Scott Emmons and the Male Wiring Project has completely elucidated the connectivity of the male and hermaphrodite nervous system.

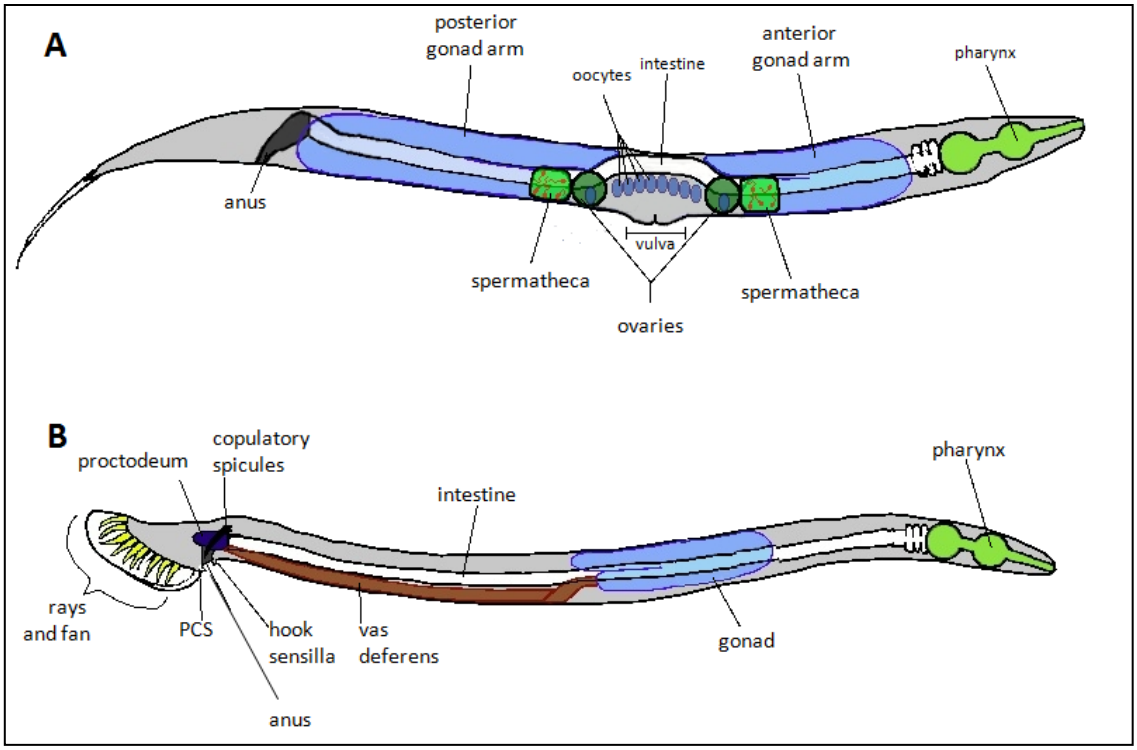


Figure 1. Sexual dimorphism in *C. elegans*.
(A) Cartoon of the major anatomical structures of the *C. elegans* hermaphrodite, and a picture of a *C. elegans* hermaphrodite *in vivo*.
(B) Cartoon of the major anatomical structures of the *C. elegans* male, and a picture of a *C. elegans* male *in vivo*.

Studies conducted by the Male Wiring Project and multiple contributing laboratories have dissected the components of the male mating circuit and established their connectivity and signaling pathways [15,16].

The small and efficient wiring of the *C. elegans* nervous system is thus an ideal environment in which to study behaviors. A behavior can be defined as the coordinated musculature, neuronal, and chemosensory outputs of an organism in response to a stimulus, either external or internal. There are many model organisms that are more complicated than *C. elegans,* and labs that study echolocation and song processing in the brains of bats, audio-visual reconstruction of the perceived environment in owls,

motivation and addiction in rodents, and even molecular changes due to exposure drugs in postmortal human brains abound [17-20]. However, these model organisms possess billions if not trillions of neurons, and attempting to dissect how their behaviors are computed and carried out on the level of individual neurons is currently impossible. The small size and extensively-studied connectivity of the *C. elegans* nervous system allow study of behavioral and motivational outputs as a result of changes in individual cells.

Additionally, *C. elegans* is cost-effective and relatively hassle-free to maintain; it eats *E. coli*, which reproduces rapidly and does not take up a large amount of space. *C. elegans* is an invertebrate with a very short generation time; it can be transported, operated on, killed, starved, and exposed to drugs, toxins, and mutagenic agents with ease, and multi-generational experiments can be completed in a short time.

Mating in male *Caenorhabditis elegans* as a model to study behavior at the neuronal level

Mating is a complex innate behavior in *C. elegans,* which is performed upon reaching adulthood. At each step of this behavior, the male must integrate the excitatory and inhibitory mechanosensory, chemosensory, and homeostatic signals received from the external environment, the hermaphrodite, and his own regulatory structures, and calculate the appropriate behavioral response at each step during the behavior. It is the most detailed and complicated behavior that *C. elegans* performs [21].

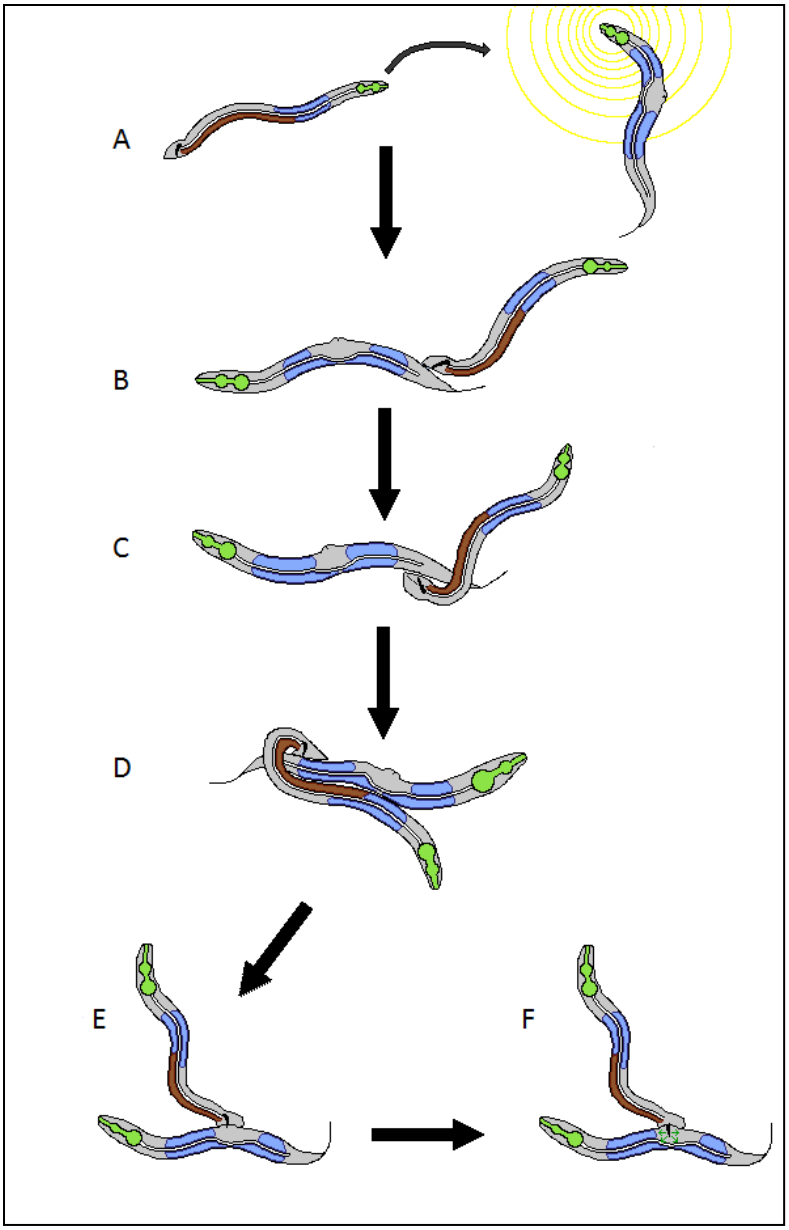


Figure 2. Cartoon depicting the steps in male mating behavior
(A) The male senses diffusible mating cues from the hermaphrodite and begins crawling toward it.
(B) The male makes contact with the hermaphrodite. Forward motion halts, and the ray sensillla in his tail mediates contact with the hermaphrodite's cuticle.
(C) The male begins scanning laterally along the hermaphrodite's cuticle, searching for the vulva.
(D) If the male reaches either end of the hermaphrodite without locating the vulva, he executes a ventral turn and continues scanning the other side of the hermaphrodite. Steps (C) and (D) are repeated until the vulva is located.
(E) The male locates the vulva. Position of the tail is confirmed by the sex-specific sensilla (rays, hook, and PCS). The male presses his tail ventrally on the hermaphrodite and begins rhythmically prodding the vulva with his copulatory spicules.
(F) Proper orientation on the vulva is confirmed by the sex-specific sensilla and the SPC neurons (SPV/SPD). Tonic protraction of the spicules drives them deep into the vulva, and ejaculation transfers sperm into the hermaphrodite's uterus. The male remains transiently associated with the hermaphrodite while secreting an inhibitory plug, then retracts his spicules and leaves.

Male mating is mediated by various neural inputs both connected to the external environment and between structures inside the worm. This behavior involves efficient usage of and cross-talk between small numbers of neurons (383 in the male) located in structures and sensilla, forming a highly cross-linked neural network throughout the anatomy of the animal. Male mating, though not as widely studied as more simple behaviors in hermaphrodites, is nonetheless an effective model for analyzing how changing environmental conditions are perceived and how they induce systemic and localized changes within a neural network (Figure 2).

Mating in *C. elegans* begins when a male worm senses diffusible cues released by sexually mature hermaphrodite worms (Figure 2A). These cues are diffusible and are sourced from the entire hermaphrodite worm, not just its vulva [22]. The male will purposely crawl towards these cues in an attempt to locate a mate.

Motivation to mate in *C. elegans* males is strong; well-fed animals will often leave a plentiful lawn of OP50 *E. coli* bacteria, its main food source *in vitro*, and actively search for a mate as the desire to mate overrides the desire to eat [23]. Physical contact of the male with the hermaphrodite has been shown to be sufficient to override further mate-searching behavior [23]. The paired RnA and RnB neurons in rays 1-6 and rays 7-9 are essential for promoting exploratory mate-searching behavior. When the male contacts a mate, the ray neurons of the male-specific sensilla sense the physical presence of the hermaphrodite, relaying a signal via interneurons to the cephalic amphid neurons, which sense food and hermaphrodite cues [24]. The cephalic amphid neurons normally inhibit mate-searching behavior by increasing affinity of the male for food [24].

The NSM neurons, which extend processes that monitor pumping rate and the presence of food in the pharynx, can attenuate or inhibit both pharyngeal pumping and excitability of the spicule protractor muscles. The UNC-103-encoded ERG-like K+ channel normally attenuates mating motivation and excitability of the neurons in the mating circuit by hyperpolarizing the spicule protractor muscles by preventing spontaneous exocytosis of vesicles containing acetylcholine from the SPC sensory-motor neurons into the synaptic cleft between the SPC neurons and the spicule protractor muscles [25,26]. The NSM pharyngeal neurons regulate LEV-11-mediated contraction rates of both pharyngeal and sex muscles through interaction with the insulin-like receptor DAF-2. LEV-11 is the *C. elegans* homolog of tropomyosin, a contractile muscle cell protein. In food-deprived conditions, the worm's pharynx pumps slower than normal. The NSM neurons sense the lower pumping rate and raise the excitability of the sex muscles. Conversely, when the male contacts a hermaphrodite's vulva, signals from the ray neurons travel to the NSM neurons, causing them to inhibit pharyngeal pumping [25].

Upon contacting the hermaphrodite, the male stops moving forward and firmly presses the ventral side of his tail, containing the ray sensilla, hook sensillum, post-cloacal sensilla (PCS), and spicules, against the hermaphrodite's cuticle (Figure 2B). The male moves laterally along the cuticle of the hermaphrodite, scanning for the vulva with his tail (Figure 2C). If he reaches the head or tail of the hermaphrodite without finding the vulva, he will execute a ventral turn and begin scanning the other side (Figure 2D). Multiple ciliated neurons, including the six IL2 neurons of the inner labial

sensilla, may play a role in detecting hermaphrodite cues before and during mating [27]. The ray neurons are extensively involved in sensation of physical contact with the hermaphrodite, and relay positional information through interneurons to the CP ventral cord motor neurons [28,29]. Rays 1, 5, and 7 mediate the male response to a dorsal contact with a hermaphrodite, while rays 2, 4, and 8 coordinate sensory-motor response to a ventral contact [28]. The RnB neurons in rays 1-6 are required to respond to the male's contact with the hermaphrodite [28], and rays 7-9 along with serotonergic signaling from the six male-specific CP (Cell body in ventral cord and Preanal ganglion) motor-neurons regulate ventral curvature of the male's tail, ensuring that it remains pressed against the hermaphrodite [11]. Dopaminergic ray neurons regulate fine motor control during turning [11]. The ray neurons are functionally redundant (Figure 3B) [14].

When his tail locates the vulva, sensed by the HOA and HOB neurons of the hook sensilla, the male halts all motion [28,29] and relies on positional input from the spicules and the three neuron pairs of the PCS to maintain positioning on the vulva [30]. Cholinergic signals are relayed from the hook and the cholinergic neurons in the post-cloacal sensilla (PCS) to the posterior sex muscles (oblique muscle, gubernaculum, and anal depressor) (Figure 3A) [31,32]. This signaling induces contraction of the oblique muscles, which are connected to the body wall, causing a ventral curvature of the tail that presses it firmly against the hermaphrodite's vulva (Figure 3A) [29]. The male then initiates rhythmic prodding of the spicules as the hook and PCS neurons communicate with spicule protractors indirectly via gap junctions to the remaining sex muscles [31,32].

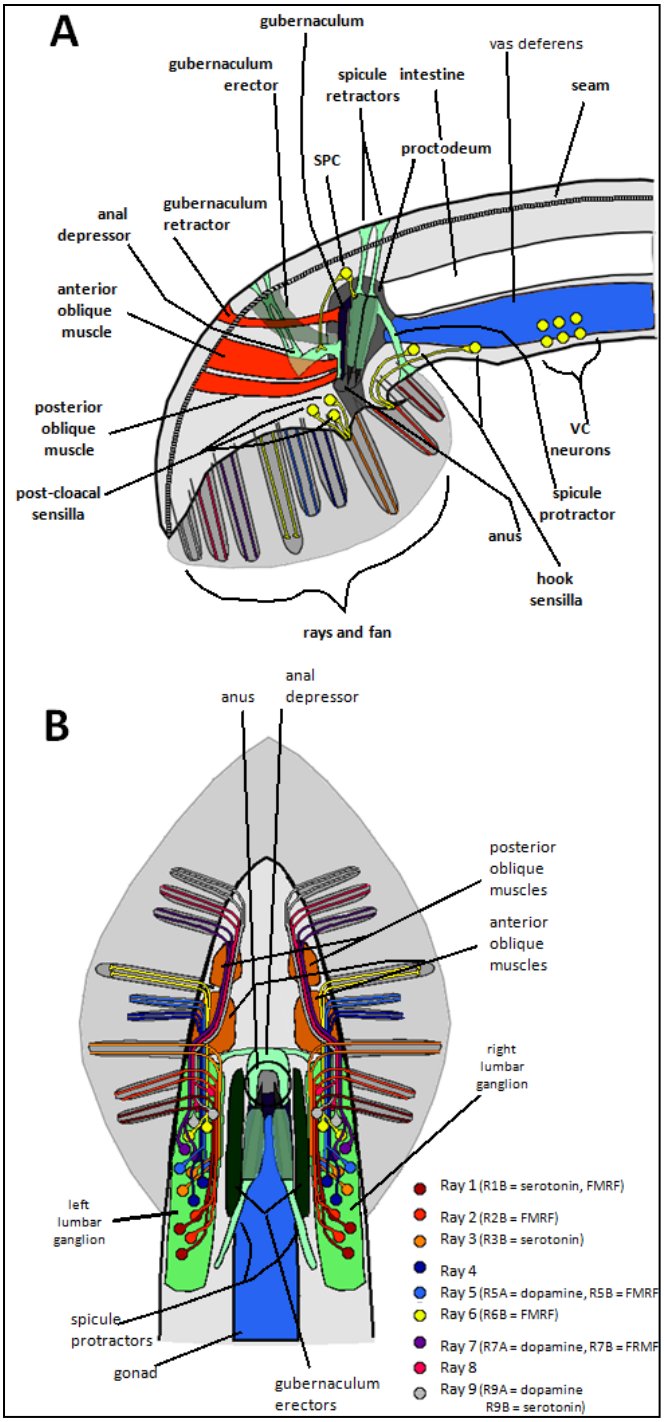


Figure 3. Gross anatomy of the sex-specific male mating apparatus.
(A) Lateral view of the *C. elegans* male tail, with sex-specific structures, neurons, and muscles
(B) Ventral view of the *C. elegans* male tail, showing sex-specific structures, neurons, and muscles, as well as the connectivity of ray neurons in their respective lumbar ganglia.

Sex muscles directly innervated by the PCS neurons include the gubernaculum erector, which regulates the position of the gubernaculum, a tapered structure that guides the spicules out of the proctodeum and aligns them with the vulva, and the oblique muscles, which maintain positioning on the vulva [29,31]. Prodding is enabled by alternating contractions and relaxations of the spicule protractor and retractor muscles, mediated by calcium currents from unc-68 ryanodine receptor calcium channels (Figure 3A) [31].

If a prodding attempt results in a partial penetration into the vulva, this information is relayed via the SPV and SPD neuron pairs to the spicule protractors, anal depressor, and gonad, and via gap junctions to the hook sensillum and PCS neurons [29]. The SPV and SPD neuron pairs thus ensure fine coordination of muscular contraction to maintain a favorable angle for spicule insertion. Signaling from these neurons induces tonic contraction of the spicule protractor muscles, mediated by *egl-19*-encoded voltage-gated calcium channels [31]. Protraction thrusts the spicules deep into the hermaprhodite's vulva, and sperm travel distally from the gonad and briefly accumulate at the proximal end of the vas deferens immediately next to the cloacal opening. The pre-ejaculatory accumulation of sperm behind the valve can be readily visualized with conventional microscopy [28,29]. This occurs in conjunction with the hypercontraction of the anal sphincter, which seals the anus shut, preventing defecation during mating, and causes the posterior section of the intestine nearest the cloaca to move dorsally. The dorsal movement of the intestine aids in opening of the valve-like structure at the distal

end of the vas deferens, permitting sperm to pass through the cloaca and be ejaculated into the hermaphrodite [29].

I used these complex *C. elegans* mating behaviors and the small, well-characterized nervous system that mediates them to study how cholinergic signaling controls a minimal neural circuit. Sustained protraction of the spicules and mating-specific tail-curvature behaviors associated with the posterior sex muscles were used as metrics to quantify my results. It has been shown that cholinergic signaling between components of the male's tail sexual anatomy help maintain and switch between behavioral states within the system [26,32]. The process of male mating behavior is understood in terms of cholinergic signaling, but very little is known about what contributions, if any, are made by cholinergic neural circuits in the cephalic region. Several of these cephalic cholinergic neurons are known to have chemosensory, mechanosensory, and sensory-motor functions in the hermaphrodite, but they have not been assessed in terms of their contributions to mating behavior. I hypothesize that a subset of these neurons sense favorable mating conditions in the external environment and relay this information to the tail, causing an increase in mating motivation.

I visualized the cephalic cholinergic neurons using the specific promoter *Punc-17*. The *unc-17* gene encodes a vesicular acetylcholine transporter (VAChT), which loads acetylcholine into presynaptic vesicles in cholinergic neurons before they are exocytosed into the synaptic cleft [33]. While reporter constructs of *Punc-17* driving fluorescent proteins express in all cholinergic neurons throughout the anatomy of the male worm, the *Punc-17small* core promoter drives expression in only the cholinergic

neurons of the head and pharynx [31,34]. I synthesized a plasmid that fuses the *Punc-17small* promoter to cDNA encoding the protein fusion ChR2::YFP, to create a fluorescently-labeled method by which I can stimulate specific neurons [35].

ChR2 is the gene for the Channelrhodopsin-2, first discovered in *Chlamydomonas reinharrdtii* by Peter Hegemann in 2002 and inserted into *C. elegans* in 2005 [35,36]. Any neuron expressing ChR2 can be selectively stimulated. The ChR2 gene encodes a seven-domain light-gated transmembrane ion channel that facilitates influx of cations into the neuron [36]. ChR2 does not respond to photostimulation unless its cofactor, all-*trans*-retinal (ATR) is bound. *C. elegans* can absorb ATR by consuming supplemented *E. coli* [35]. ATR absorbs high-intensity light and forms a double-bond in its long-chain carbon tail (Figure 4A). The molecule isomerizes from *trans*-retinal to *cis*-retinal [36]. Isomerization induces a conformational change in the ChR2 channel to which ATR is bound, mediating the opening of the ion channel and the influx of cations (Figure 4B). The neural membrane is rapidly depolarized and the neuron fires [36].

A recent study found that a spectrum of mating-like ventral tail curvature could be induced by direct Channelrhodopsin-2-mediated stimulation of individual ray neurons. Varying degrees of ventral tail-curving (which they termed “sickle” and “hook”) were induced by ChR2-mediated direct stimulation of either three A-type (cholinergic) neuron pairs in rays 3, 4, and 6, or by three A-type dopaminergic neuron pairs in rays 5, 7, and 9, or by a combination of eight B-type and three A-type neuron pairs.

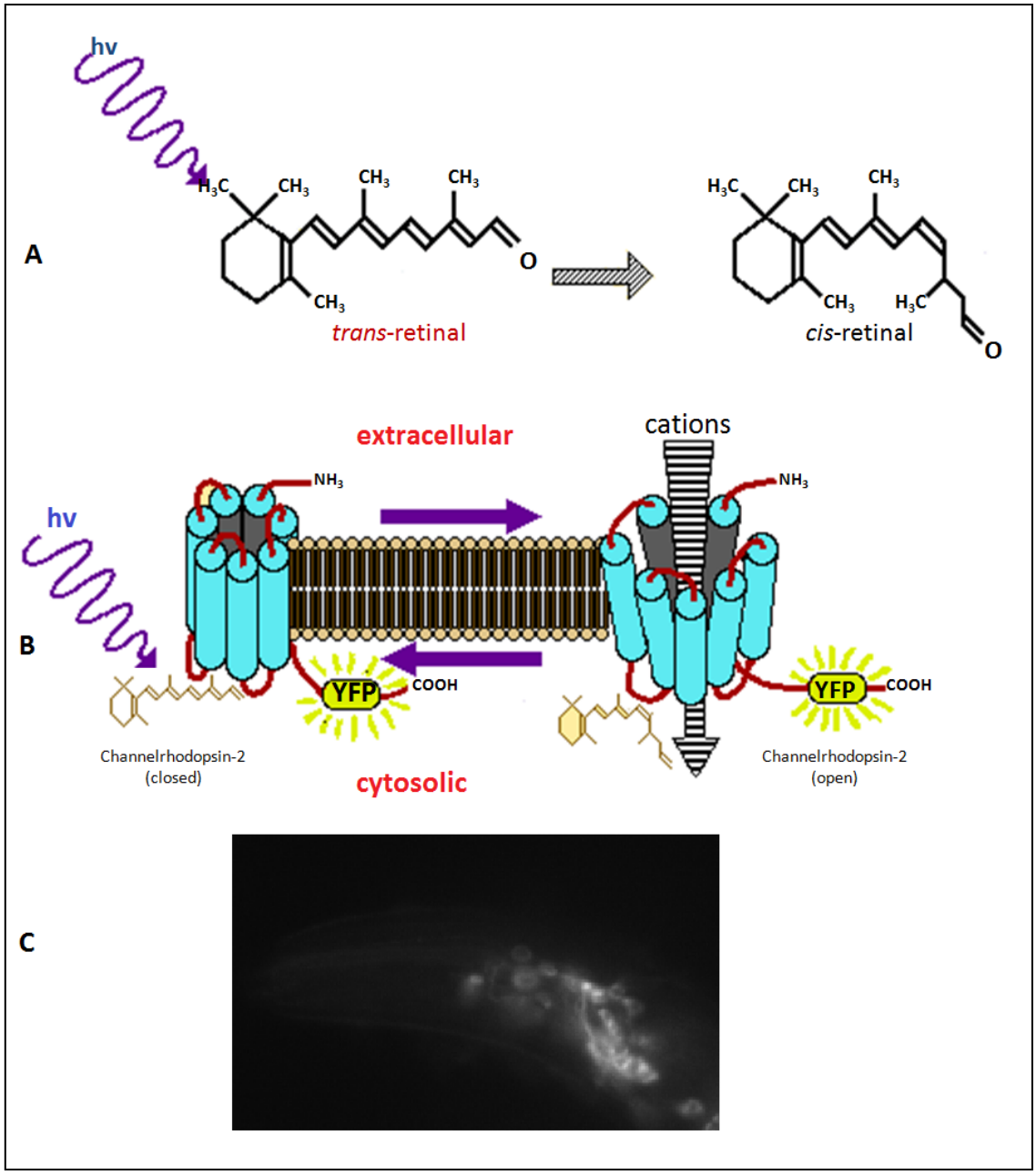


Figure 4. Structures of and interaction between Channelrhodopsin-2 and all-*trans*-retinal.
(A) Isomerization of all-*trans*-retinal (ATR) in response to light energy
(B) Structure of Channelrhodopsin-2 and conformational change in response to isomerization of ATR. The central pore of the ion channel is closed until incident light causes *trans*-retinal to isomerizes to *cis*-retinal, inducing a conformational change that opens the channel and allows an influx of cations.
(C) Photograph of cephalic cholinergic neurons labeled by PJM1 (*Punc-17small:ChR2::YFP)* construct. 40X – GFPA fluorescence

Tail-curling (which they termed "circinate") was induced by ChR2-mediated direct stimulation of either three B-type neuron pairs in rays 1, 2, and 3, or the eight B-type neuron pairs found in eight of the worm's nine rays [14]. These behaviors resembled the ventral curvature induced in the male's tail by the sex muscles as he mounts and maintains position over the hermaphrodite's vulva.

Specific aims

The objective of my thesis was to demonstrate a novel role of the cephalic cholinergic neurons in male *C. elegans*. I sought to show that selective stimulation of these neurons generates a signal that has the capacity to regulate the excitability of the mating-specific circuits in the tail and causes precocious display of mating-like behaviors. I ruled out the possibility that the blue light-stimulated behaviors could occur in hermaphrodites or *in vivo* in males without a high-intensity stimulus. I showed that propensity to display these behaviors post-stimulus positively correlated with age of the worm, which concurred with previous studies [37]. I identified a subset of cephalic cholinergic neurons that are responsible for generating and relaying an uncharacterized cholinergic signal from the head to the tail. I analyzed how direct stimulation of these neurons affected the sensitivity of the spicule-protraction circuit to various acetylcholine agonists, and mating motivation as measured by a potency assay. Furthermore, I showed that stimulation of the cephalic cholinergic neurons partially rescued the decline in ACh

agonist-mediated spicule protraction and mating potency associated with food deprivation.

CHAPTER II

EXPERIMENTAL PROCEDURES

Strains

All worms contained the *pha-1(e2123)* allele on linkage group III (LGIII), the *him-5(e1490)* allele on LGV, and the *lite-1(ce314)* allele on LGX [8,10,38]. The strains used in the course of this study were: *pha-1, lite-1, him-*5N2; rgIs12 and rgEx551 (*pJM1, pha-1, lite-1, him-5(;* and *pLR160, pha-1, lite-1, him-5.* All strains were maintained on NGM agar plates seeded with OP50 according to [4]. The *pha-1, lite-1, him-5* strain was maintained at 15^{o}C, and all injected strains were maintained at 20^{o}C. Pharmacological assays were conducted at 20^{o}C. DNA microinjections, laser ablations, and behavior assays were all conducted at 21-23^{o}C.

Creation of the pJM1 plasmid

The Gateway LR clonase (Invitrogen) procedure was used to generate the pJM1 (*Punc-17small*:ChR2::YFP) from PLR159, which contained the Punc17small promoter flanked by AttL recombinations sites with ampicillin resistance, and PLR167, which contained the gene for chlorampicillin resistance flanked by AttB sites and proceeded by the ChR2::YFP fusion. The LR clonase enzyme allowed fast recombination, placing the *Punc*-17small promoter in front of the ChR2:YFP fusion. This construct was

transformed into competent DH5α *E. coli* cells and purified using the Qiagen Miniprep protocol.

Microinjection

Injected plasmids (pJM1 and pLR160) were diluted in sterile H_2O to a concentration of 50ng/uL, and co-injected with 50ng/uL of the pBX1 plasmid and 100ng/uL of the pUC18 plasmid, into a strain containing the *pha-1(e2123)* allele as an injection marker, the *lite-1* allele, and the *him-5* allele. This mixture was injected into both gonadal arms of a virgin adult hermaphrodite's using a Narishige Model MMO-203 microinjector with an Eppendorf Femtojet air compressor, mounted to a Zeiss Axiovert 200 compound microscope on a Kinetic Systems Vibraplane air table to minimize vibrations. Injected worms were then incubated at 20^{o}C on NGM agar plates seeded with OP50.

The *pha-1(e2123)* allele is a temperature-sensitive mutation that arrests development in the pharynx, preventing the late terminal differentiation and morphogenesis of all pharyngeal cell types during embryogenesis [39,40]. At 25^{o}C, the mutation is 100% embryonic lethal. At 15^{o}C, affected worms appear wild-type and their pharynxes develop normally. In successfully injected hermaphrodites, the *pha-1(e2123)* loss-of-function mutation would be rescued by the pBX1 plasmid, and progeny would survive at 25^{o}C.

Successful injection of the *Punc-17small:ChR2::YFP* construct was confirmed by YFP fluorescence in the cephalic cholinergic neurons expressing ChR2::YFP. Fluorescent virgin L4 F1 hermaphrodite progeny were separated onto individual OP50 plates and allowed to give birth to an F2 generation. If the parent hermaphrodite was successfully injected, the pJM1 and pBX1 plasmids would be passed on to the F1 generation, and present in both their sperm and eggs. The F1 generation would self-fertilize, giving rise to stable F2 lines with males and hermaphrodites both containing the pJM1 plasmid, as indicated by fluorescence of the cephalic cholinergic neurons. The injected line was saved by freezing and given the designation rgEx551.

The anatomic location of the ChR2::YFP construct in the cholinergic head and pharynx neurons was visualized with fluorescence microscopy at 480nm wavelength, using the HCImage program that controlled a Hamamatsu EM-CCD ImageEM digital camera mounted to an Olympus BX51 compound microscope, using an OLYMPUS U-LH100HGAPO GFP filter and cross-referenced with [41]. Injected Channelrhodopsin-2 fused with YFP does not always express stably or identically in neurons (Figure 4C). Thus, pLR160 (*Punc-17small*:*CFP*) was co-injected at a concentration of 50 ng/uL using the same procedure above to visualize the anatomic localization of the cholinergic head and pharyngeal neurons (Figure 5).

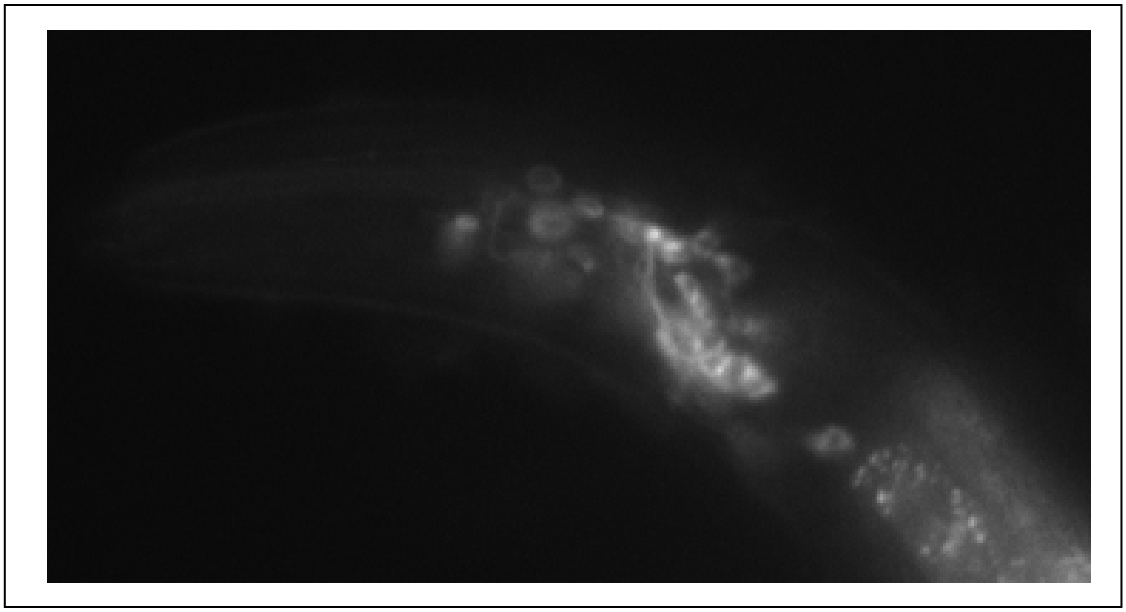


Figure 5. Expression Pattern of pLR160 (*Punc-17small::CFP*) in cephalic cholinergic neurons
This is a composite of four optical planes of the head of an rgIs12 day-1 adult male *C. elegans* worm. YFP fluorescence, 100X.

Optical stimulation of transgenic worms

Virgin sexually immature worms were selected at L4 stage. Worms that were selected for ATR exposure were raised overnight on NGM agar plates seeded with a lawn of OP50 *E. coli* bacteria supplemented with 50mM all-*trans*-retinal (ATR). All plates supplemented with ATR were covered with aluminum foil to prevent unwanted exposure of the ATR to ambient light. Worms not intended for ATR exposure were selected at their sexually immature L4 stage and raised on NGM agar plates seeded with OP50 without any ATR. Worms destined to be starved and stimulated were selected at the L4 stage and raised overnight on NGM plates seeded with OP50 supplemented with 50mM ATR, then transferred to a bacteria-free NGM agar plate with a pipette filled with

distilled water to remove attached bacteria. They were then moved to NGM plates on which 20-uL of 50mM ATR, dissolved in distilled water, was allowed to soak into the agar. Worms destined to be starved, but not stimulated, were raised overnight on NGM agar plates seeded with OP50 without ATR, and then transferred via the same method to NGM agar plates on which a 20-uL droplet of water was allowed to soak in to the agar. All plates were ringed with 100% glycerol, creating a high-osmolarity barrier that repelled worms from crawling up to the side of the plate and desiccating. This treatment has not been shown to alter worm behavior in any other way [26].

All behaviors were digitally recorded for a total of 190 seconds using the SimplePCI video imaging program that controlled a Hamamatsu ImagEM Electron multiplier (EM) CCD camera attached to an Olympus SZX16 stereomicroscope. When worms destined to be stimulated reached the first day of adult stage, they were given a 10-second stimulus of high-intensity blue light from an EXFO X-Cite 120PC Q Fluorescence Illumination System filtered by an SZX2-FGFPA GFP filter (Ex460-495/Em510-550) and then exposed for three minutes to low-intensity white light. Worms that were repeatedly analyzed for behavior during aging were moved to fresh OP50 NGM plates supplemented with 50mM ATR after each filming period. Worms not receiving any stimulation were filmed for an identical 190-second period, but the initial 10-second blue-light stimulation was omitted.

Mating-like behavior assay

All males were selected from non-crowded NGM agar plates seeded with OP50. All behaviors were observed by filming with the SimplePCI digital imaging program controlling a Hamamatsu ImagEM Electron multiplier (EM) CCD camera attached to an Olypmus SZX16 stereomicroscope. Mating-like behaviors were defined as any of the subset of mating behaviors triggered by stimulation of the ray neurons [14]. An event started the moment that the tail of the worm began to curl ventrally, and ended when the tail returned to its normal anatomical position. These behaviors were divided into two subsets: full behaviors were events in which the ventral portion of the male tail, containing the spicules, post-cloacal sensilla (PCS) and the hook sensillum, curled ventrally and made physical contact with the ventral posterior third of the animal; partial behaviors were abortive events in which ventral tail-curling was observed, but ventral posterior tail contact was not made (Figure 6).

Latency was calculated by subtracting the time of onset of filming from the time of the first behavior displayed. Time spent self-scanning was calculated by determining the number of seconds during the 190-second filming time that a worm's tail was both (a) continuously in contact with his cuticle and (b) scanning forward and backward as is searching for the vulva as a percentage of total filming time. Comparisons of mean number of displayed behaviors between groups were analyzed using the Wilcoxon Ranked-Sum test.

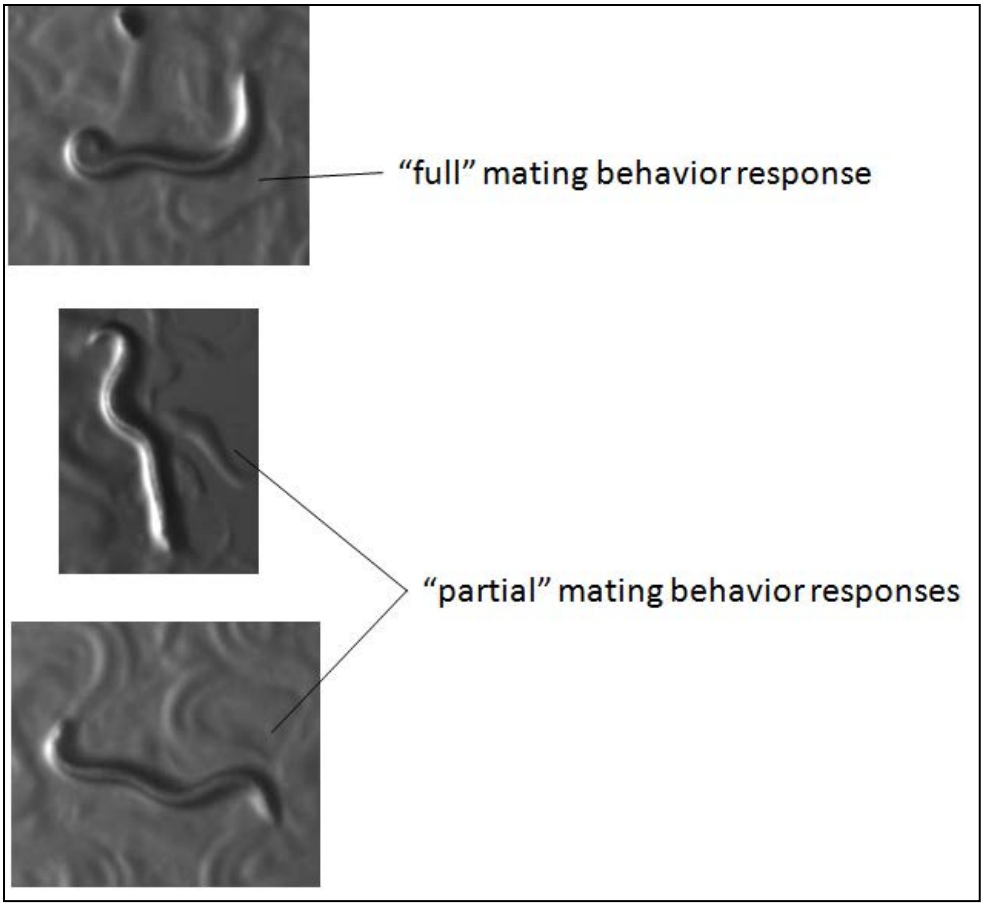


Figure 6. "Full" ("Circinate") and "Partial" ("Sickle") mating-like behavior responses to blue-light stimulation.
Images are of rgIs12 day-1 adult male *C. elegans* worms crawling on OP50 lawns at 10X. "Full" behaviors are defined by physical contact of the ventral surface of the tail with the male's ventral cuticle. "Partial" behaviors are defined as any degree of spontaneous precocious mating-like ventral tail curvature without contact with the ventral cuticle.

Integration of the pJM1 (*Punc-17:ChR2::YFP*) construct

The pJM1 plasmid did not uniformly or stably express in the same subset of cholinergic head neurons in every worm. To stably integrate the pJM1 construct into the strain's genome, worms were mutagenized with 4, 5', 8-trimethylpsoralen (TMP) using a modification to the procedure of [42]. 2,000 gravid hermaphrodite rgEx551 (pJM1, *pha-1*, *lite-1*, *him-5*) worms were selected onto large NGM plates seeded with OP50 and

incubated at 20°C overnight. They were then washed from the plates with sterile M9 buffer and placed into 15-mL conical tubes and collected at the bottom of the tube via gravity centrifugation. This procedure was repeated twice more, and the hermaphrodites were then suspended in three times their own volume of 30ug/mL 4, 5', 8-trimethylpsoralen (TMP), covered in foil, and incubated at 20°C for 15 minutes. The worms were poured along with the TMP into an empty large petri dish and irradiated with UV light for one minute at an intensity of 340 $\mu W/cm^2$, activating TMP. TMP cross-links DNA, triggering nucleotide excision repair mechanisms and infrequent recombination events in the cells of the TMP-exposed worms. If an injected extrachromosomal array is recombined into the worm's genome during one of these recombination events, it will be stably integrated and will pass uniformly from generation to generation [42]. Each of these hermaphrodites was then moved to an individual NGM agar plate seeded with OP50. The worms were permitted to reproduce for two weeks, and their progeny were then screened. 8 plates in which the cholinergic head neurons of all worms fluoresced were selected as candidates for successful genomic integration of the pJM1 construct.

To remove unwanted mutations caused by TMP, virgin L4 *pha-1; him-5; lite-1* hermaphrodites from each plate were selected and incubated overnight on an NGM plate seeded with OP50, on which virgin *pha-1*, *lite-1*, *him-5* males were placed. After one day of mating, all adult worms were removed and the F1 generation was allowed to mature synchronously to adulthood. Virgin L4 hermaphrodites were selected from this population and again paired with virgin L4 *pha-1, lite-1, him-5* males. Out-crossing to

pha-1, lite-1, him-5 males was repeated a further four times. The integrated homozygote was extensively expanded to fresh NGM agar plates seeded with OP50 to generate a working population, and was then frozen and given the designation rgIs12.

Laser ablations

All laser ablations followed the protocol established in [43]. Laser ablations of single neuron pairs were carried out in *pha-1*, *lite-1*, *him-5* males at L3 stage using a Spectra-Physics VSL-337ND-5 nitrogen laser (Mountain View, CA) attached to an Olympus BX51 microscope. All males were selected from non-crowded NGM agar plates seeded with OP50. Subjects were mounted alone on a 2% noble agar pad containing 5.0mM sodium azide (NaN_3) as an anesthetic agent. NaN_3 uncouples proton transport through the F_0 subunit of ATPase, inhibiting ATP synthesis [44]. Care was taken to minimize exposure of the animal to the anesthetic. To control for behavioral results of exposure to NaN_3, each experimental subject was paired with an L3-stage control male, which was mounted on an identical 2% noble agar pad with 5mM NaN_3 for an identical time period, but was not laser-ablated. The anatomical location of the IL2, IL2V, and IL2D neuron pairs were visualized by fluorescence of the ChR2::YFP construct controlled by the Punc-17small promoter (Figure 7). Neuron pairs were ablated first distally, then proximally so as to minimize collateral damage to surrounding tissues.

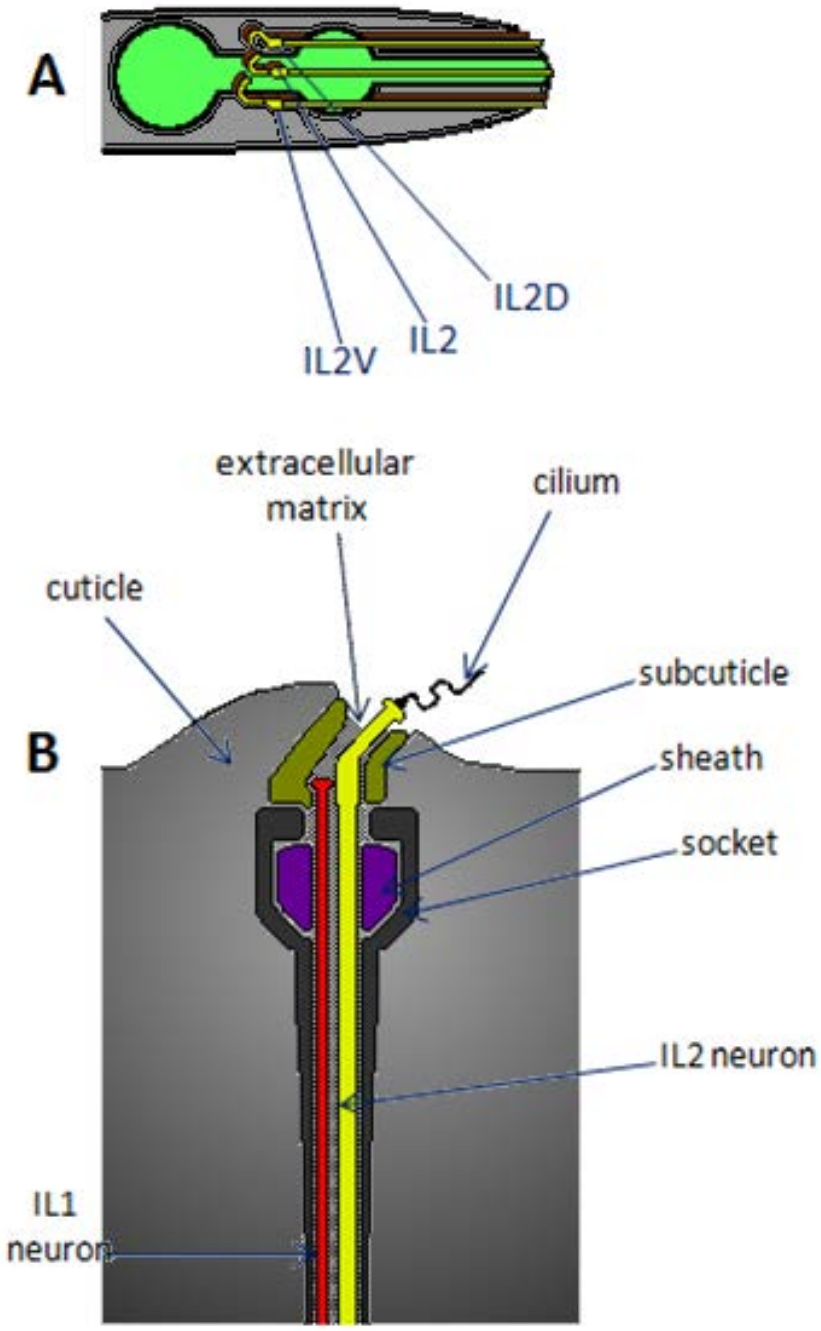


Figure 7. Anatomy of the inner labial sensilla.
(A) Location of the six IL2 neurons of the inner labial sensilla behind the anterior pharyngeal bulb.
(B) Fine anatomy of the dendrite of an IL2 neuron as it extends through the cuticle and contacts the external environment. The six IL2 neurons are morphologicallyidentical. IL2 neurons are the only ciliated cholinergic neurons in the head of the male *C. elegans* worm. The cilium extend through a hole in the cuticle, and may be involved in chemosensation. The IL1 neurons also form part of the inner labial sensilla, but they are not cholinergic and they terminate inside the cuticle.

Both the ablated and the control groups were given a 2-day recovery period at 20^{o}C on NGM agar plates seeded with OP50. Ablation of these neuron pairs caused many subjects to wander aimlessly around the plate, so a 100% glycerol ring was used to repel worms from the plate walls. Each worm was transferred to a 100% glycerol-ringed NGM plate seeded with a 20-uL bolus of OP50 with 50mM ATR, covered in foil, and allowed to uptake the retinal for a minimum of one hour before stimulation and filming as described above.

Pharmacology

Levamisole (LEV) (purchased from ICN Biomedicals, Inc., Aurora, OH), nicotine (NIC) (purchased from EM Industries,Inc., Gibbstown , NJ), and arecoline (ARE) (purchased from Indofine Chemical Company, Hillsborough, NJ) were dissolved in distilled water to make 100mM stock solutions and stored at -20^{o}C. These were serially diluted in 1mL aliquots to appropriate concentrations in distilled water. EC_{50} and EC_{90} were determined for nicotine (NIC) (0.1µM, 10µM, 500µM, 1000µM), levamisole (LEV) (1.0 nM, 10nM, 100nM, 1.0µM, 2.0µM), and arecoline (ARE) (1.0µM, 10µM, 100µM, 1.0mM, 10.0mM). 1mL of the appropriate drug was dispensed into a well on a nine-depression Pyrex spot plate. All males were selected at the L4 stage and incubated at 20^{o}C overnight on NGM agar plates seeded with OP50, and moved to a clean NGM agar plate when they reached the first day of sexual maturity. Worms were moved in groups of 10 directly to the well via a pipette with distilled water. Virgin males destined

to be stimulated were selected in groups of 10 at the first day of sexual maturity from this population and moved to NGM agar plates seeded with OP50 supplemented with 50mM ATR. These were wrapped in foil and kept at 20^{o}C for a minimum of one hour, after which the entire plate was given a 10-second stimulus of high-intensity blue light as described above. All worms were removed as soon as possible after stimulation and transferred to the wells via a pipette with distilled water. Virgin males destined to be starved were selected from the same population as above, moved to a clean NGM agar plate and allowed to crawl around for 2 minutes to remove attached bacteria, then transferred to another clean NGM agar plate and starved for 1 hour. All plates were ringed with 100% glycerol to prevent worms from crawling up the walls and desiccating.

After being placed in the appropriate drug, males were observed continuously for 5 minutes using a Leica MZ 7.5 stereomicroscope and assayed for sensitivity to the concentration level and type of drug used. A male was considered 'sensitive' to a given drug if, at any point during the 5-minute observation window, it fully and continuously protracted its spicules for at least 5 consecutive seconds. Worms that partially or intermittently protracted their spicules were not counted as sensitive (Figure 8). After 30 males were assayed in a single well, the plate was washed with distilled water and dried, and then a fresh 1mL aliquot of the appropriate drug was added to the well. All comparisons of the mean percent sensitivity between groups were analyzed using Fisher's exact test.

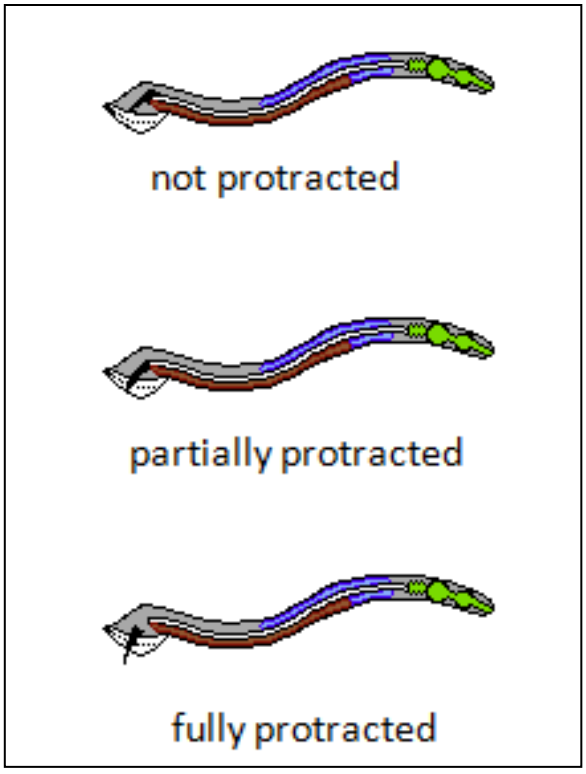


Figure 8. Cartoon depicting protraction of a *C. elegans* male's copulatory spicules.
The spicules must protrude fully through the cloaca to be considered fully protracted.

CHAPTER III

RESULTS

Stimulation of cephalic cholinergic neurons induces mating-like behaviors

Previous studies showed that the spicule-protraction circuit in the male *C. elegans* tail is regulated by cholinergic signaling between the PCS neurons and the posterior male sex muscles [32]. For the male worm to initiate protraction of his copulatory spicules, the PCB and PCC neurons in the PCS must sense physical contact with the hermaphrodite's vulva and relay a cholinergic signal to the anterior and posterior oblique muscles, which are connected to the lateral body wall of the male worm, as well as a cholinergic signal to the spicule muscles [29]. Tonic contraction of the oblique muscles induces a ventral curvature in the male tail, stabilizing the male's location at the vulva during the rhythmic contraction of the spicules initiated by cholinergic signaling from the PCS [32].

The input of cholinergic signaling from cephalic neurons has not yet been studied. I hypothesized that, by sensing cues from the external environment, a subset of these neurons determine favorable mating conditions and transmit a signal to the mating circuits in the tail, increasing their excitability. To examine this, I first labeled all cephalic cholinergic neurons by microinjecting the pJM1 construct (containing the *unc-17small* promoter regulating transcription of fusion of the Chr2 and YFP genes) as

described above. I then photostimulated the ChR2::YFP-expressing cephalic cholinergic neurons to examine the behavioral results thereof.

The *unc-17* gene encodes a highly-conserved vesicular acetylcholine transporter responsible for pumping acetylcholine into pre-synaptic vesicles [33]. The *unc-17* promoter can drive expression of various transgenes in all cholinergic neurons within the animal [34]. Previous experiments have identified a truncated form of the promoter, *Punc-17small*, which can drive fluorescent protein expression in all cholinergic neurons found within the head and pharynx [31].

A group of fluorescent virgin L4 rgEx551 males (n=17) was selected and prepared for mating behavior assay using the methods described above. A control group of virgin L4 rgIs12 males (n=20) was identically prepared. Upon stimulation, the males of the experimental group became highly motive, and displayed stereotyped behaviors in their tails which resembled the ventral curvature induced by contraction of oblique muscles during mating (Figure 9A). Several types of behaviors occurred as a result of stimulation. During the stimulation timeframe, responsive males often linearly stiffened the posterior one-half of their bodies. This stiffening halted sinusoidal crawling motions in this section of the animal, causing the rear half of the worm to be dragged behind the rest of the animal as it crawled, carving a wide trough in the lawn of OP50 (Figure 9A). Tail stiffening was often followed by varying degrees of spasmic ventral curvatures of the tail, although these curvatures also occurred before or in the absence of tail stiffening.

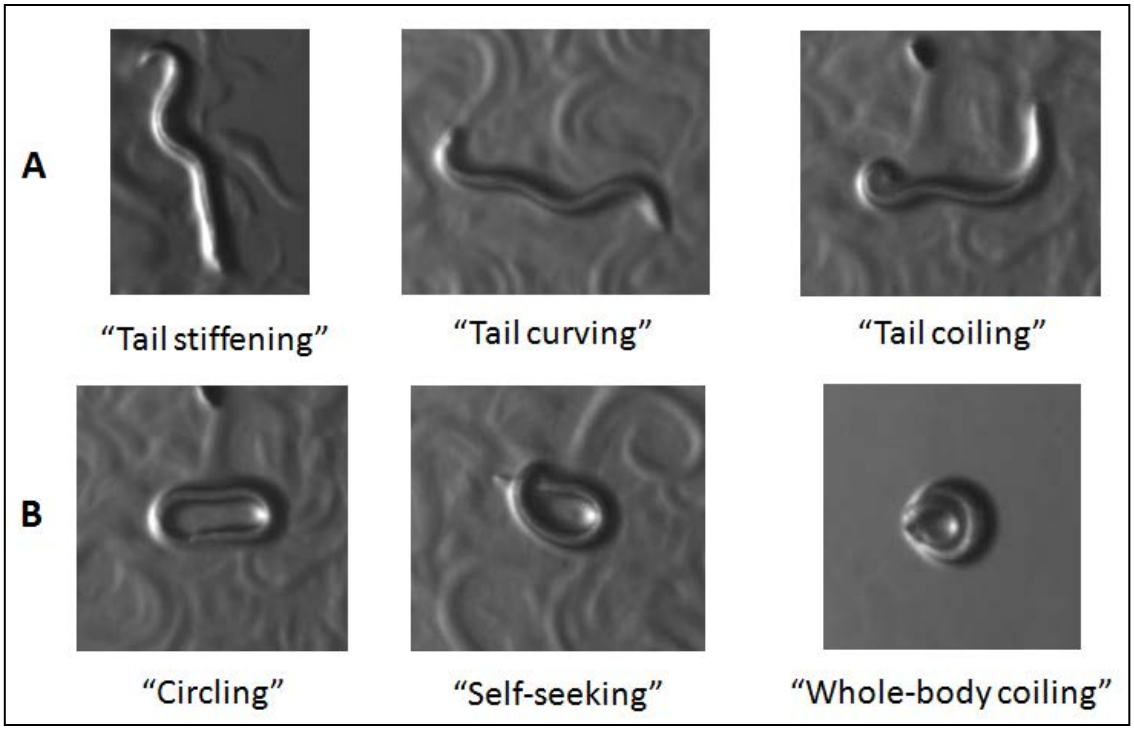


Figure 9. Subtypes of mating-like behaviors displayed after blue-light stimulation.
(A) Mating-like responses, including tail stiffening, tail curving, and tail-coiling, mirroring what was observed in [14]. These are *de novo* responses that have not been observed *in vivo* in wild-type *C. elegans*.
(B) Idiosyncratic responses, including circling, self-seeking, and whole-body coiling. Though interesting, these have been observed in wild-type worms on NGM agar plates. They are not counted in this study.

The tail either curved ventrally but failed to contact the ventral cuticle of the worm, or curled completely, contacting the cuticle (Figure 9A). This ventral curvature resembled the ventral curvature induced by dorsal ventral contraction of the anterior and posterior oblique muscles against the lateral body wall during copulation [29]. Similar kinds of ventral tail curvature known to be mating-specific have been induced by specific ChR2-mediated stimulation of the RnA and RnB ray neurons [14]. Furthermore, during and after stimulation, physical contact between the male's tail and any other part of his body, whether incidental or as a result of spasmic tail-curling, caused the animal to begin a stereotyped "self-seeking" behavior, in which the male tail moved ventral

laterally or posterior laterally on his cuticle, as well as turned and reversed course when it reached the animal's mouth/pharynx. This behavior resembled lateral motions that the male makes when scanning for the vulva on a hermaphrodite [28]. If the male's tail contacted the anterior pharyngeal region, it appeared to press down and adhere to the opening of the pharynx, and the worm moved in a circular motion as long as the tail remained in contact with the mouth/pharynx (Figure 9B). These circular motions often progressed into self-seeking behavior. Finally, in many cases where a worm displayed self-seeking behavior, the animal would often coil up tightly upon itself, and its tail would spasm rhythmically, pressing tightly against its ventral cuticle (Figure 9B).Wild-type male *C. elegans* worms are known to occasionally display self-seeking, circling, and body-coiling behaviors naturally [28]. For this reason, the increase in frequency of these idiosyncratic behaviors, while interesting, was not analyzed further in the course of this study.

The spasmic ventral tail-curving and tail-coiling behaviors, however, have not been demonstrated to occur in wild-type worms *in vivo*. I decided to use the number of these behaviors displayed as a metric to analyze the results of stimulating cholinergic neurons labeled by the pJM1 (*Punc-17small:ChR2::YFP*) construct on the male mating circuit. The mating-like behaviors in the tail continued long after the incident blue light was turned off. The experimental group displayed a mean of 2.5±0.58 (Student's t-test, P=0.0005) of these behaviors during the 190-second filming period on the first day of adulthood, while the control worms, which had the *lite-1* allele, did not exhibit any reaction to the blue-light stimulus (Figure 10).

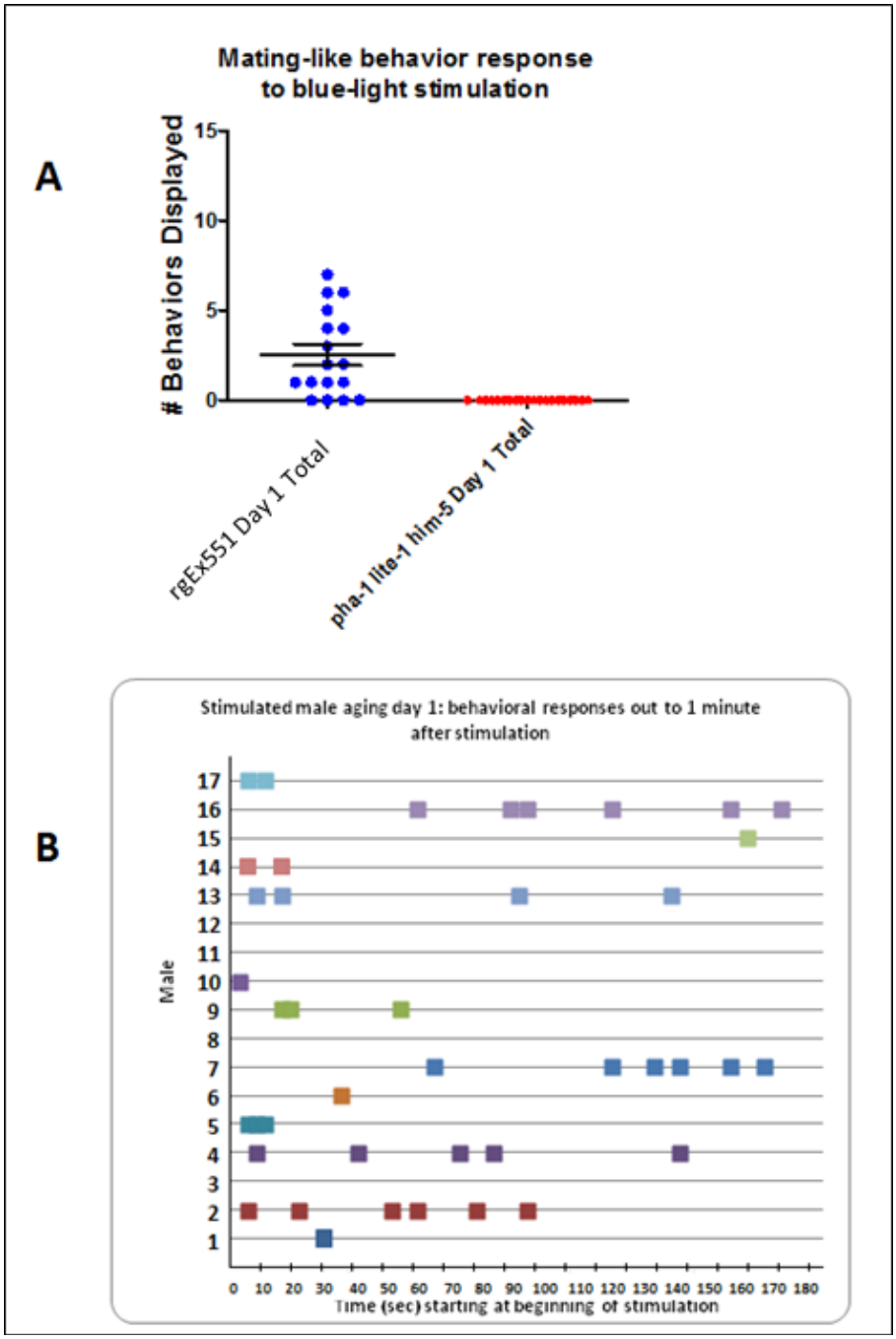


Figure 10. Mating-like behaviors displayed in response to direct ChR2-mediated stimulation of cephalic cholinergic neurons.
(A) Responses of worms with the PJM1 (*Punc-17small:ChR2::YFP*) construct vs uninjected *pha-1, lite-1, him-5* worms.
(B) Time course of behavioral responses by worms with the PJM1 (*Punc-17small:ChR2::YFP*) construct. Each horizontal line represents an individual 1-day old adult male worm. Each square represents a mating-like behavior displayed at the time indicated on the *x*-axis.

Since the ventral tail-curving and tail-curling behaviors appeared to be related to mating, and mating in male *C. elegans* is known to decay as the animal ages, the stimulated and control groups were further analyzed as they aged over the next five days [37]. It was hypothesized that as the animal aged, the mating circuit would become hypo-polarized, resulting in easier and earlier firing of the neurons involved. Therefore, the number of mating-like behaviors displayed would increase with each day of aging. Each male in the experimental and control groups was set up for a mating behavior assay as described above. The experimental group displayed 3.8±0.61 mating-like behaviors on the second day of filming (Student's t-test, $P<0.0001$), 5.1±0.51 mating-like behaviors on the third day of filming (Student's t-test, $P<0.0001$), 5.3±0.67 mating-like behaviors on the fourth day of filming (Student's t-test, $P<0.0001$), 6.3±0.67 mating-like behaviors on the fifth day of filming (Student's t-test, $P<0.0001$), and 5.6±0.37 mating=like behaviors on the sixth day of filming (Student's t-test, $P<0.0001$). None of the males in the control group displayed a mating-like behavior in response to blue light stimulus during any of the six filming periods (Figure 11).

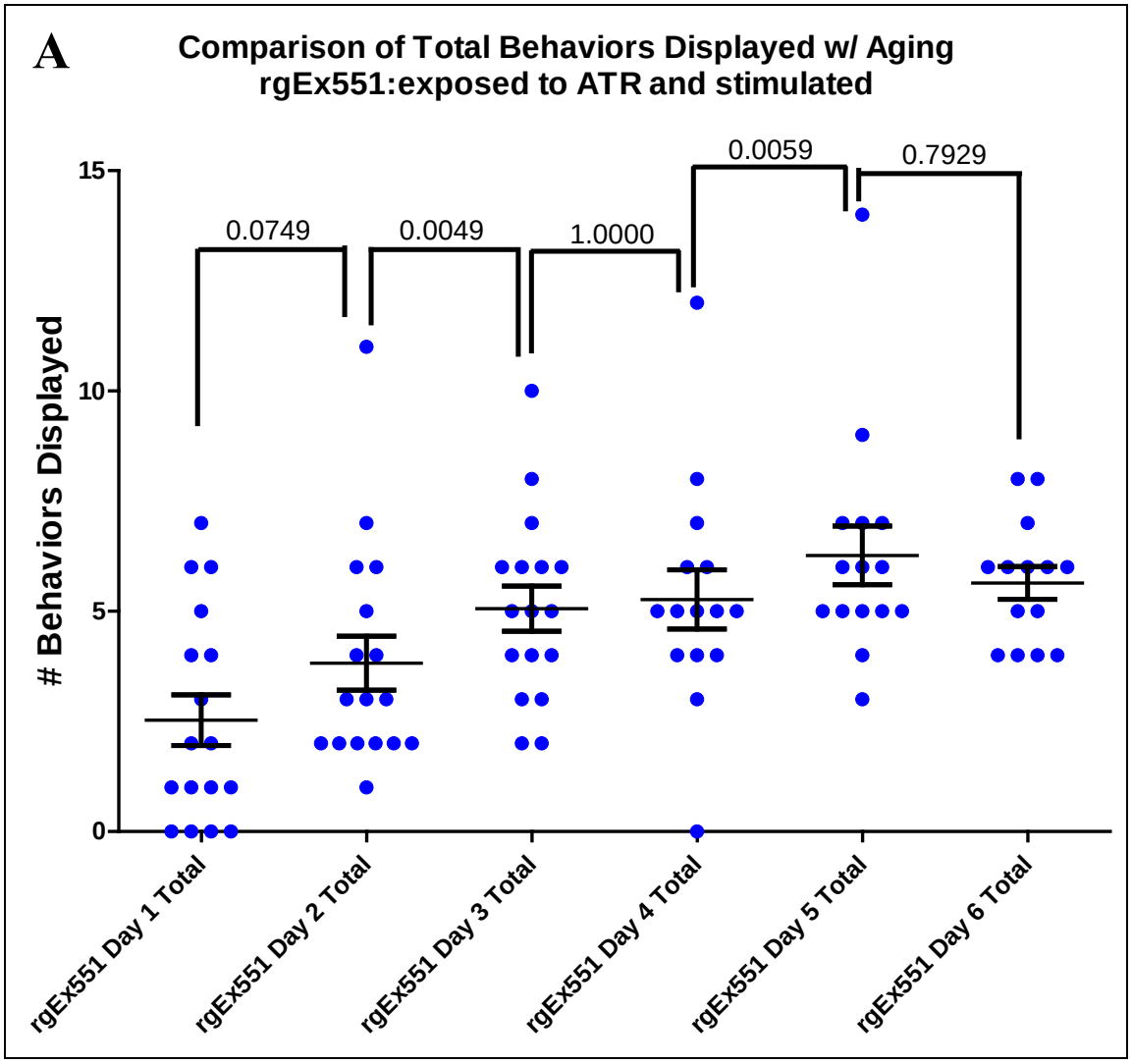
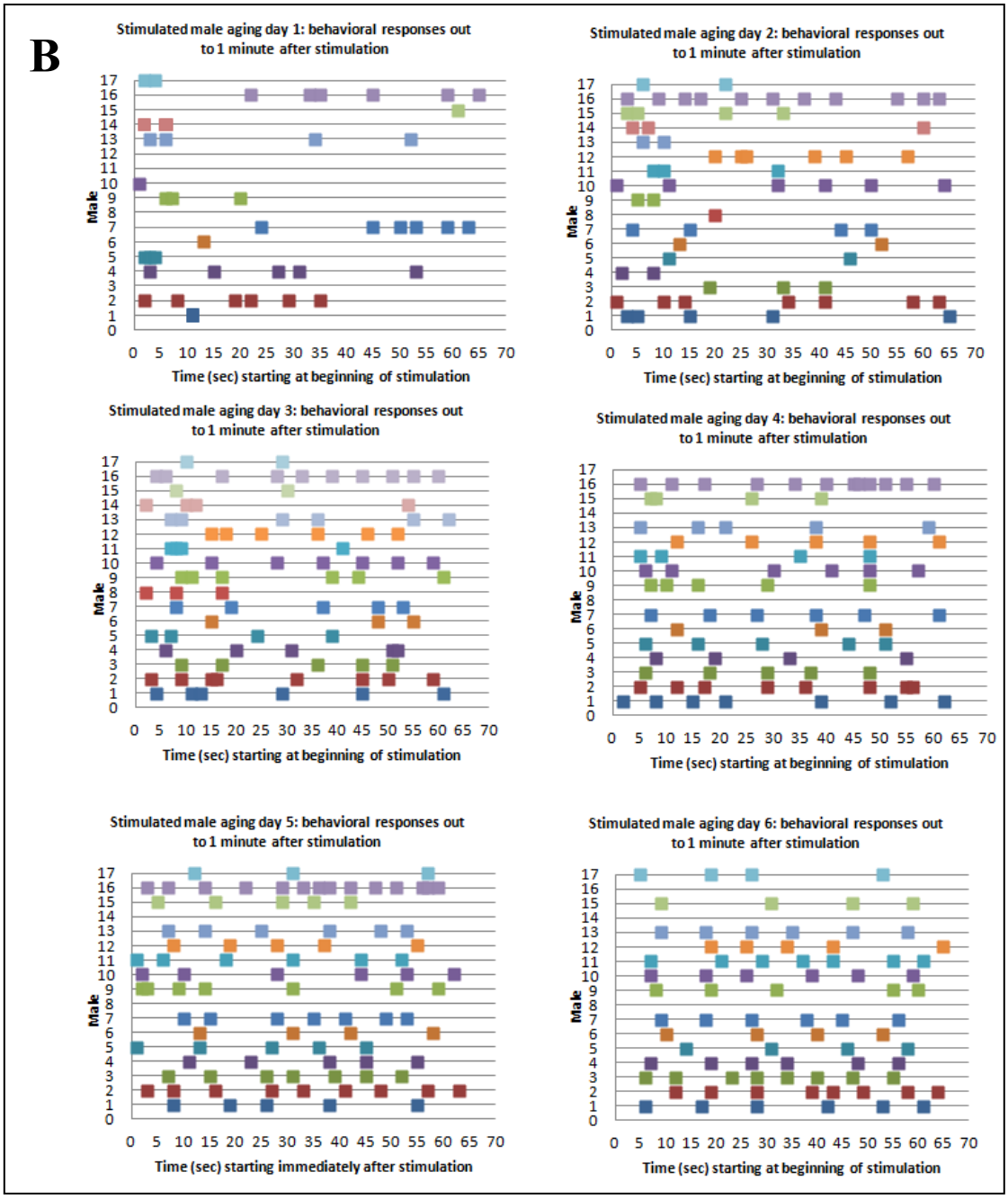


Figure 11. Mating-like behavior responses of rgEx551 males to blue-light stimulation.
(A) Mean number of mating-like behaviors displayed by the rgEx551 line compared to the age of the worms. Each blue dot represents the number of mating-like behaviors displayed during a filming period consisting of 10 seconds of stimulation with high-intensity blue light followed by 180 seconds of observation on a low-intensity white-light background. Means are compared using the paired t-test.
(B) Time courses of mating-like behaviors displayed by rgEx551 males relative to their age. Each horizontal line represents an individual male worm, and each square represents a unique mating-like behavior event at the time indicated by the *x*-axis.
(C) Mating-like behaviors displayed by the control group of *pha-1, lite-1, him-5* males with aging. Each dot represents the number of mating-like behaviors displayed by a single-*pha-1, lite-1, him-5* worm during the 190-second filming period.



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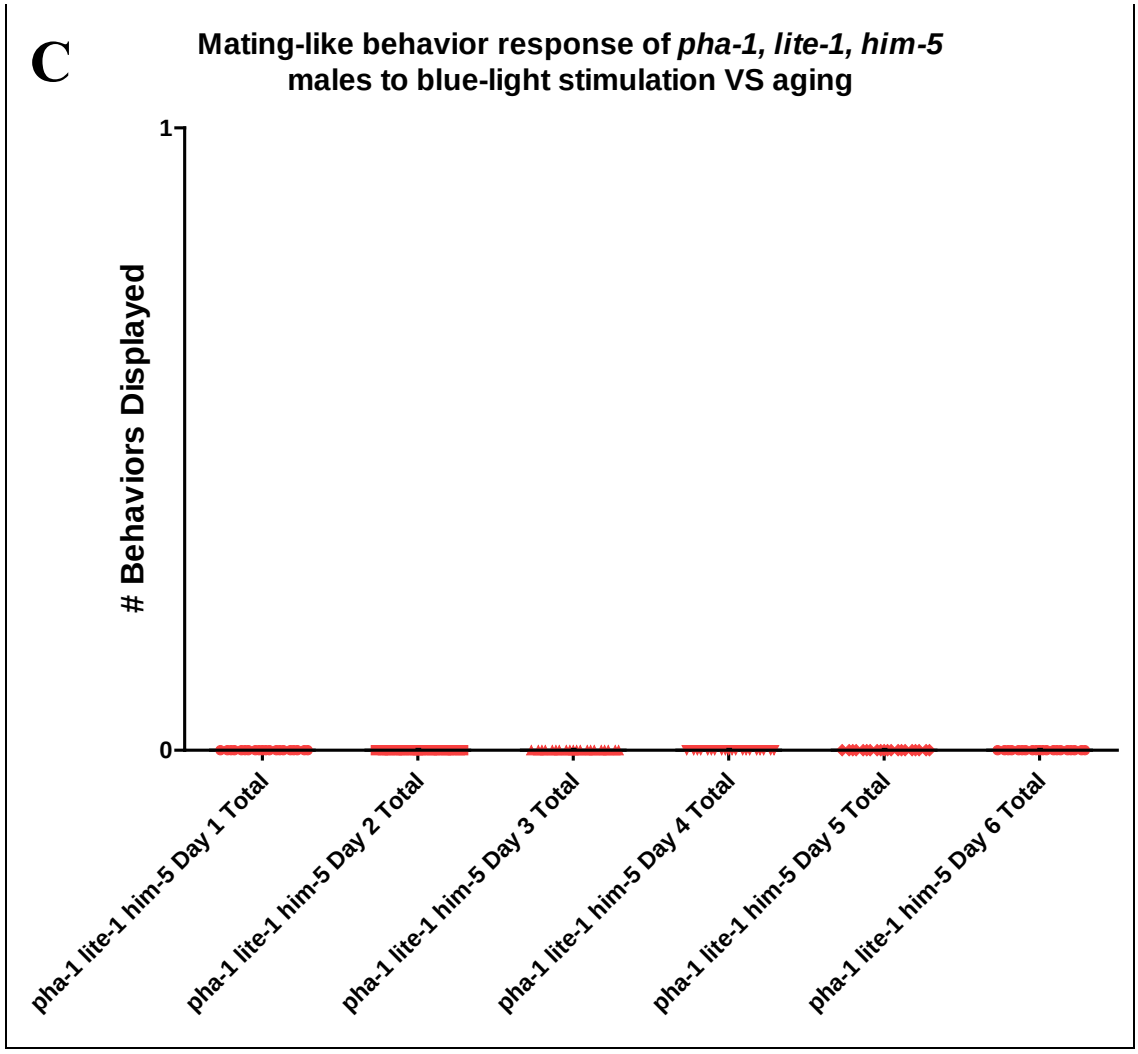


Figure 11 Continued.

The rgEx551 strain did not express ChR2::YFP in the same neurons or with the same intensity in each worm (Figure 12). To achieve uniform expression, the plasmid was integrated into the worm's genome via TMP-mutagenesis and homozygosed following the procedure described in [42]. The rgIs12 strain exhibited uniform fluorescence in the same set of 41 cholinergic neurons in the head and pharynx of the worm (IL2, IL2D, IL2V, URAD, URAV, URB, SMDD, SMDV, SAAV, RMD, RMDD, RMDV, SAAD, SIAD, SIAV, SIBD ,SIBV, SMBD, SMBV, AIY and SABV neuron pairs, and the individual SABD neuron) (Figure 12). The PJM1 construct expressed uniformly in the same set of neurons in both rgIs12 males and rgIs12 hermaphrodites.

To verify that integration of rgEx551 neither alter the expression of ChR2::YFP in cephalic cholinergic neurons nor changed the blue light-stimulated mating-like behavior phenotype, the rgIs12 (n=26) was analyzed for mating behaviors displayed with aging using the procedure described above. Ventral tail-curving and tail-curling behaviors were counted together. A control group of *pha-1, lite-1, him-5*worms (n=30) was also analyzed. The mean-number of mating-like behaviors displayed after stimulation increased significantly in rgIs12 males (Figure 13A). There was not a significant difference between the mean number of mating-like behaviors displayed by the extrachromosomal and integrated lines until the fourth day of adulthood (Figure 13B).

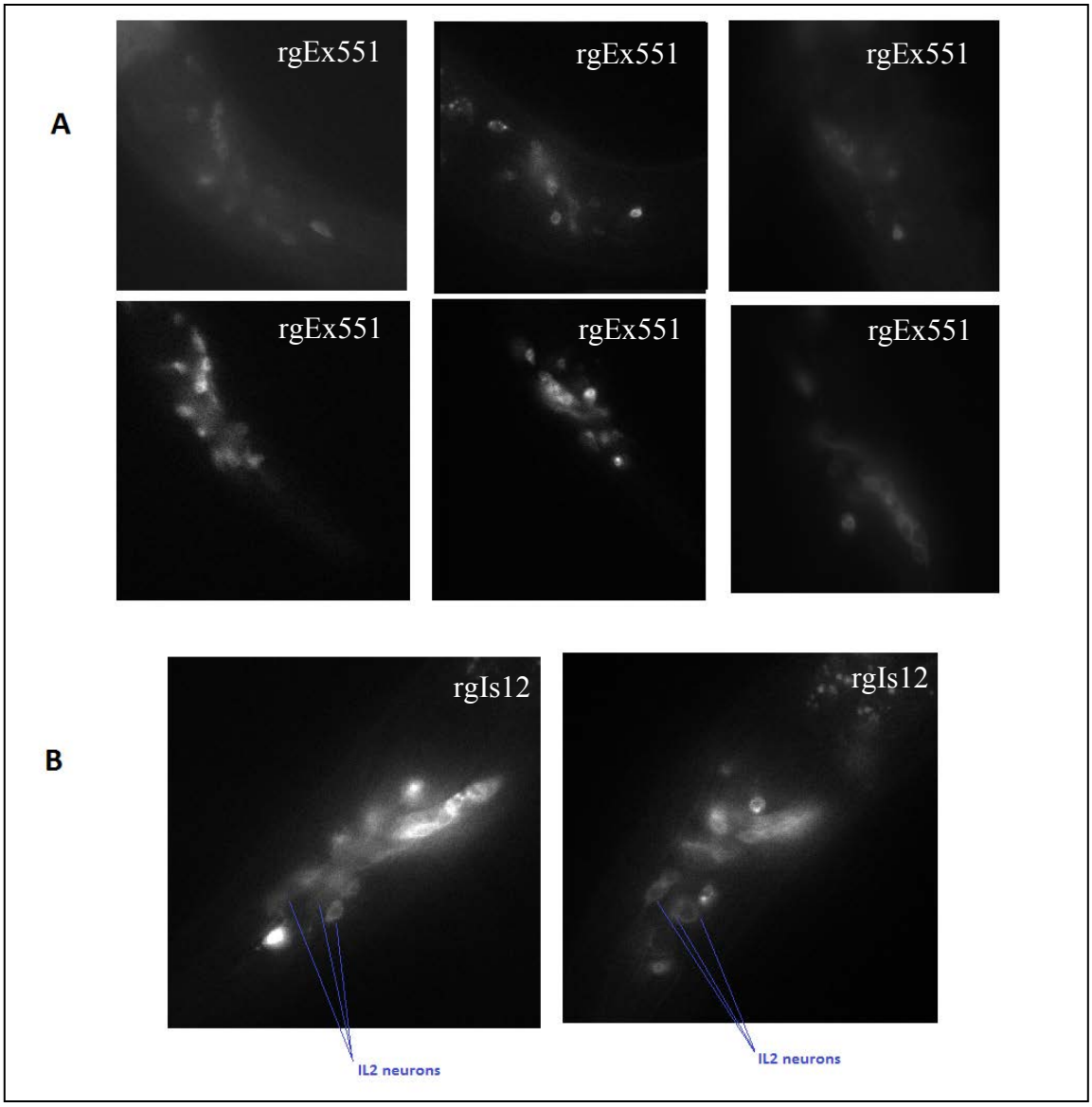


Figure 12. Differences in expression pattern between injected (rgIs12) and integrated (rgEx551) strains.
(A) Examples of varying mosaic expression of the PJM1 (*Punc-17small:ChR2::YFP*) construct between individual males in the injected (rgEx551) strain. YFP fluorescence, 100X magnification.
(B) Integration of the transgene has stabilized expression in the rgIs12 strain. The three IL2 neuron pairs forming the inner labial sensilla have been labeled. YFP fluorescence, 100X magnification.

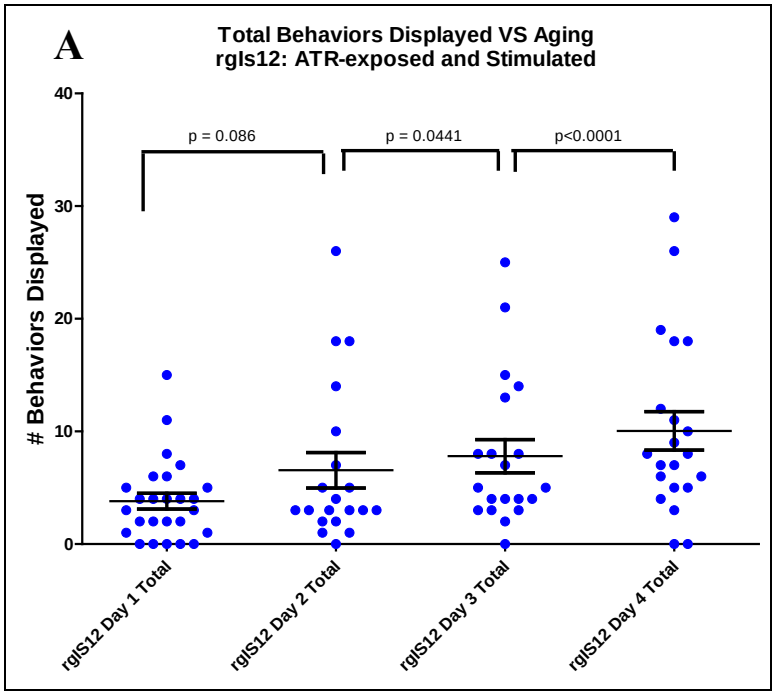
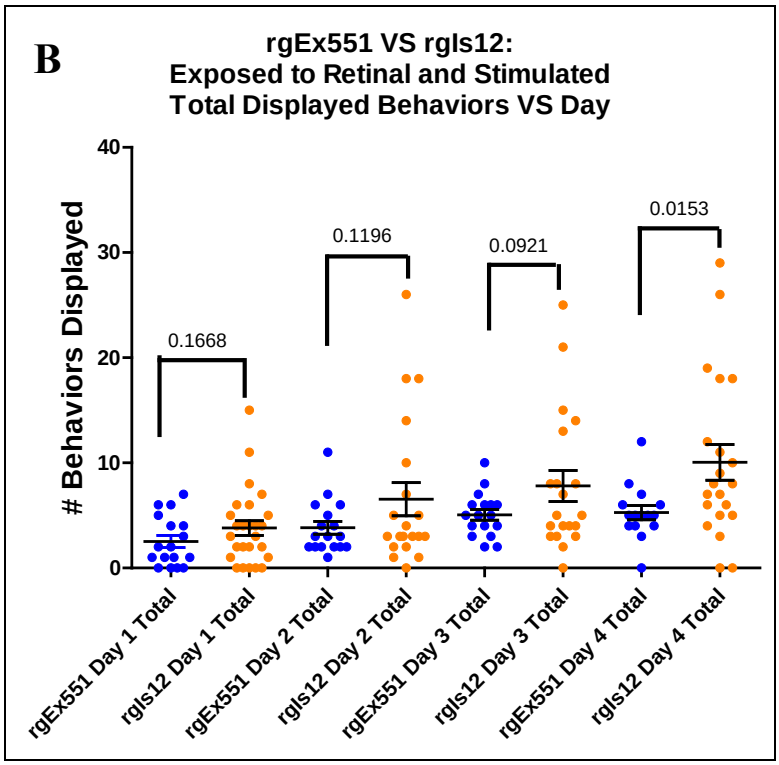
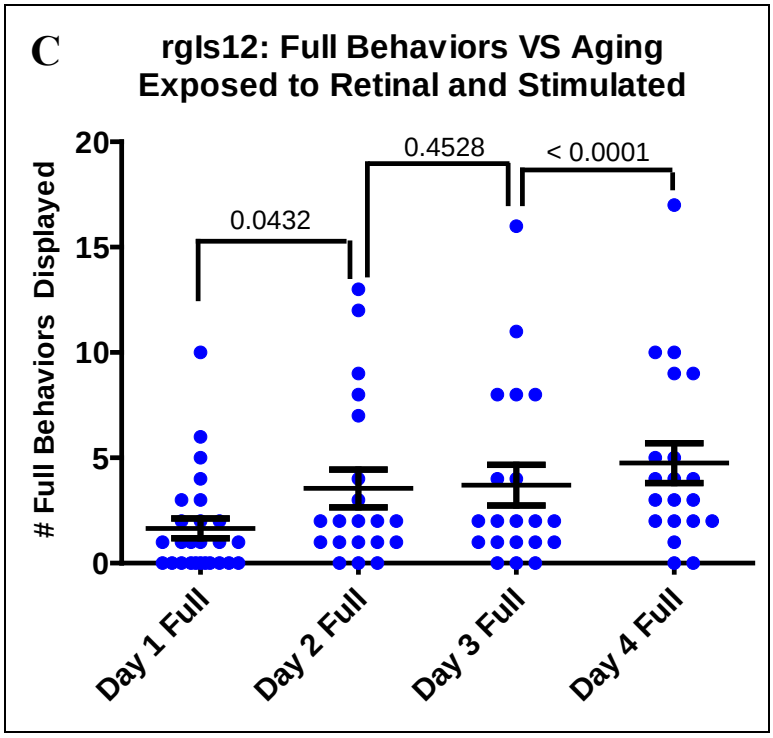


Figure 13. Comparison of mating-like behavior responses to blue-light stimulation between rgIs12 and rgEx551 strains.
(A) Total mating-like behaviors displayed after blue-light stimulation by the rgIs12 strain. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. Means were analyzed using the paired t-test.
(B) Comparison of total mating-like behaviors displayed after blue-light stimulation between the rgIs12 and rgEx551 strains. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. Means were analyzed using the unpaired t-test with Welch's correction.
(C) Full mating-like behaviors displayed after blue-light stimulation by the rgIs12 strain. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. Means were analyzed using the paired t-test.
(D). Partial mating-like behaviors displayed after blue-light stimulation by the rgIs12 strain. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. Means were analyzed using the paired t-test.





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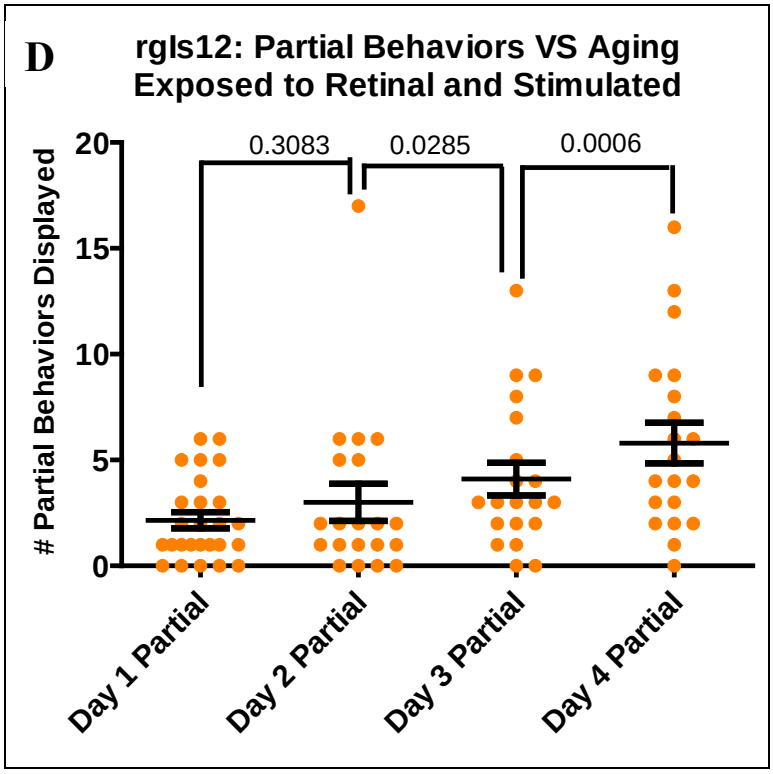

Figure 13 Continued.

The blue light-induced mating-like behaviors displayed during and after stimulation of the rgEx551and rgIs12 lines can be further classified into two sub-categories: ventral tail-curving ("sickle" or "hooked") and ventral tail-curling ("circinate") [14]. The data from the mating-like behavior assay from the rgIs12 line was analyzed using both sub-categories of behavior as separate metrics.

The mean number of ventral tail-curving behaviors did not increase significantly with age until day 4 of adulthood (Wilcoxon Ranked-Sum test, $P<0.05$) (Figure 13D). However, the mean number of tail-curling behaviors increased significantly with age (Figure 13C).

I hypothesized that these two subtypes of behaviors represented a dose-dependent response of the male mating circuit to the amount of stimulus received by the cephalic cholinergic neurons. If true, then the tail-curving behaviors would represent a "partial" response in the tail to the unfiltered white-light background, which contained a small blue-light component in its spectrum. This low level of blue light would be sufficient to open less of the Channelrhodopsin-2 channels in the cephalic cholinergic neurons. The tail-curling behaviors would represent the "full" response of the tail to the high-intensity blue (480nm) light stimulus used to trigger the ChR2 in the cholinergic head neurons.

I tested this hypothesis by using the mating-like behavioral assay on a second group (n=23) of day-1 virgin male rgIs12 worms, but replaced the 10-second blue light stimulus with an additional 10 seconds of exposure to the low-intensity white light background. I analyzed the difference in means of displayed "full" and "partial" behaviors as a result of exposure to high-intensity blue light. The mean number of "partial" behaviors displayed did not significantly differ between the stimulated and un-stimulated groups until day 4 of adulthood (Wilcoxon Ranked-Sum test, $P<0.05$). The mean number of "full" behaviors displayed was significantly different at each day of aging between the stimulated and un-stimulated groups (Wilcoxon Ranked-Sum test, $P<0.05$) (Figure 14A). Because the partial mating-like behaviors were the same whether or not a stimulus was applied, I decided to only use full mating-like behaviors, in which the male's tail curled ventrally and made contact with the ventral cuticle, as a metric for further study (Figure 14B).

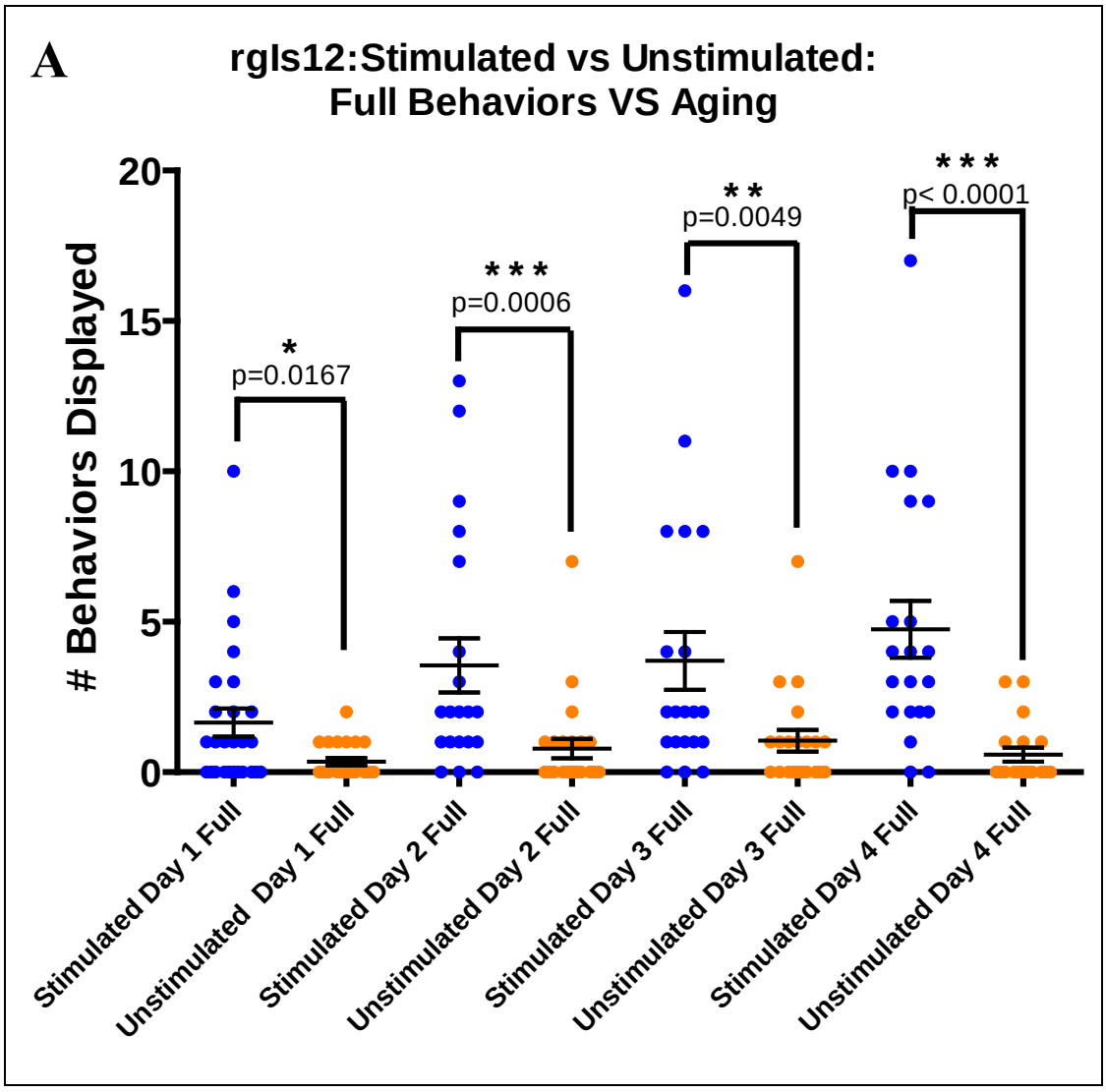

Figure 14. The effect of a low-intensity background on "full" and "partial" mating-like behaviors in stimulated and unstimulated rgIs12 males.
(A) Comparison of "full" mating-like behaviors displayed VS aging between stimulated or unstimulated rgIs12 males. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. "Stimulated" males (blue dots) were exposed to the same mating behavior assay treatment as described above. "Unstimulated" males (orange dots) were also filmed for 190 seconds, but the 10-second blue-light stimulus exposure was replaced with an additional 10 seconds of exposure to white background light. Means were analyzed using the Wilcoxon rank-sum test.
(B) Comparison of "partial" mating-like behaviors displayed VS aging between stimulated and unstimulated rgIs12 males. Each dot represents the total number of mating-like behaviors displayed by a single male worm during a 190-second filming session consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a white-light background. "Stimulated" males (blue dots) were exposed to the same mating behavior assay treatment as described above. "Unstimulated" males (red dots) were also filmed for 190 seconds, but the 10-second blue-light stimulus exposure was replaced with an additional 10 seconds of exposure to white background light. Means were analyzed using the Wilcoxon rank-sum test.

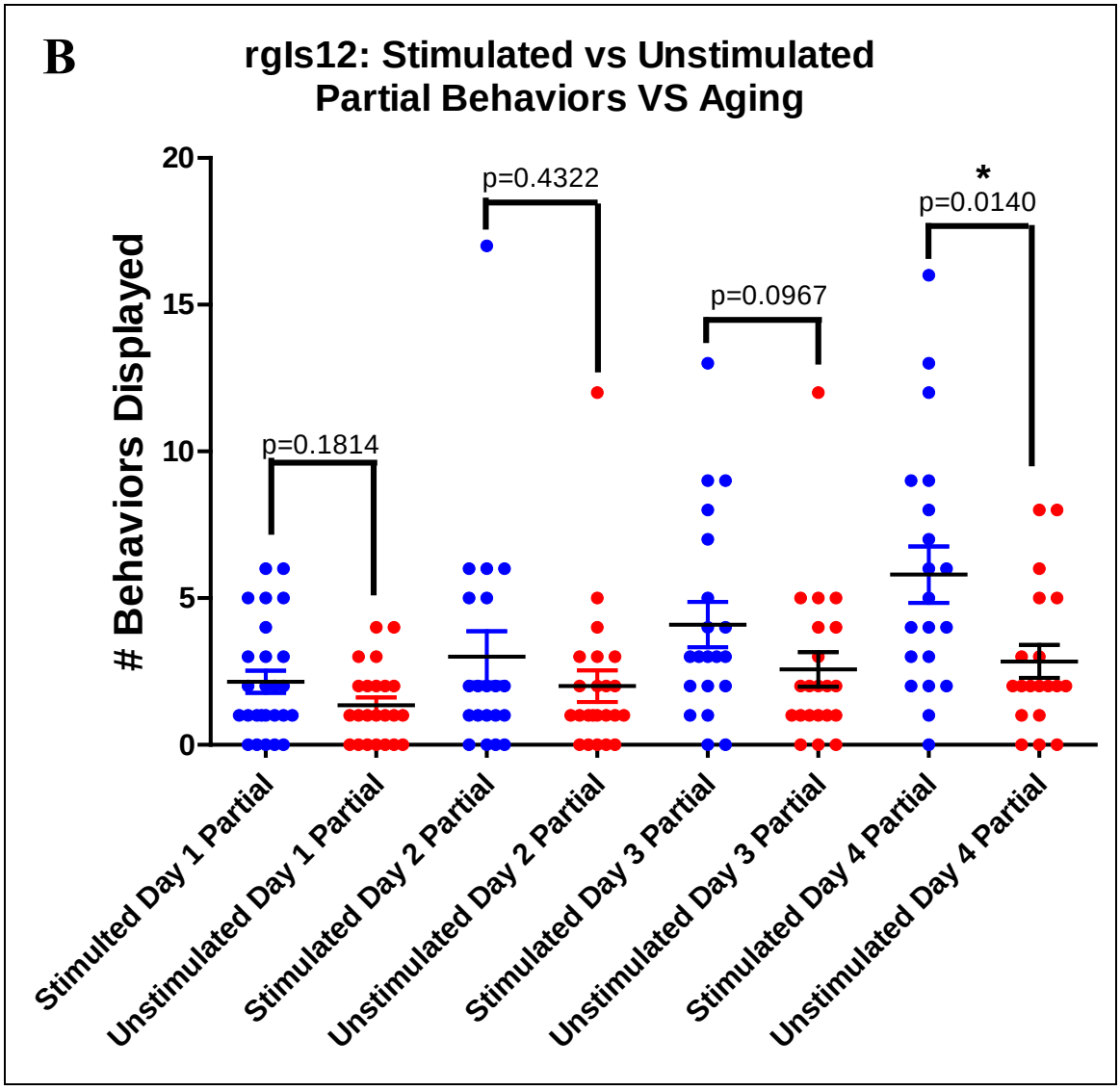

Figure 14 Continued.

There is an observable latency between stimulation and mating-like behavior response

Wild-type *C. elegans* hermaphrodites release one or more diffusible cues that act as strong chemoattractants for males [22]. These compounds have been identified as members of the ascaroside class of glycolipid signaling molecules, which are highly-conserved among diverse nematode species [45-48] If a male receives an ascaroside hermaphrodite cue and remains committed to crawling toward it for a long period of time, then this prolonged motivational state change must be mediated by some form of a memory of the reception of the cue retained within the male's mating system. I hypothesize that a subset of the cephalic cholinergic neurons in the male receive a pro-mating signal externally and then relay that signal to the mating circuits, increasing their excitability. The males in my mating-behavior assay are only stimulated for 10 of the 190 seconds during which they are filmed. If they tend to display their first mating-like behavior after the stimulating blue light has been shut off, then they have retained memory of the stimulus within either their cephalic cholinergic circuits or posterior mating circuits.

Males were analyzed using the calculation for latency as described above. The mean latency of partial behaviors decreased significantly as the worm aged (Figure 15). The mean latency of full behavior display did not statistically differ among different filming periods on different days (Figure 15).

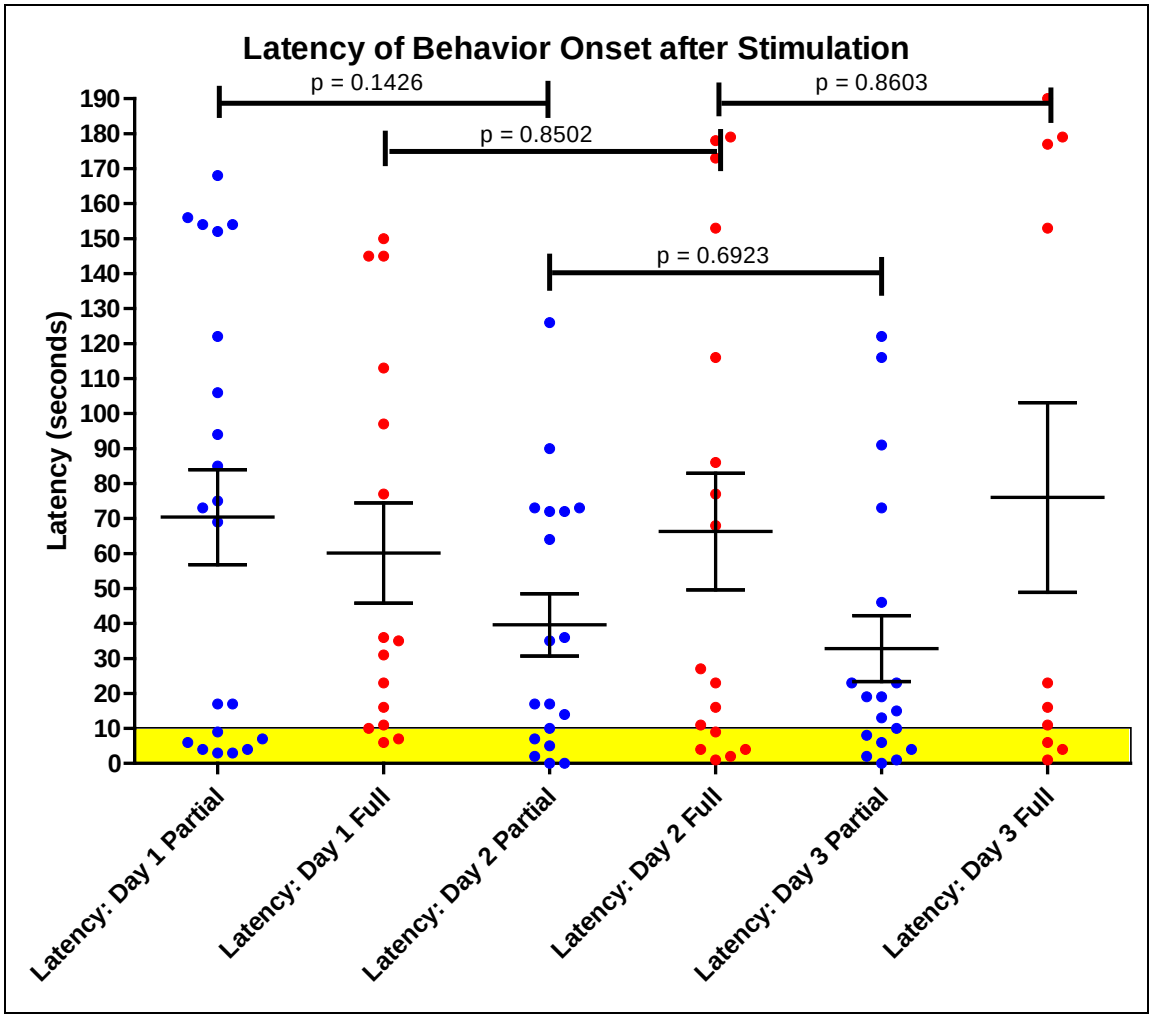

Figure 15. Latency between onset of stimulus and onset of full or partial mating-like behavior display VS aging in rgIs12 males.
Each dot represents the time difference (Latency in seconds) between the onset of stimulation (t=0) and the time (sec) of the first behavior type displayed. The yellow bar encompasses the 10-second blue-light stimulation period. Means were compared using the Wilcoxon Rank-Sum test.

The mating-like behaviors displayed after ChR2-mediated stimulation of cephalic cholinergic neurons are not observed in hermaphrodites

Although the male *C. elegans* worm possesses a unique set of muscles and copulatory structures in its tail, it was possible that the ventral tail-curvature was a non-

sex-specific response of the general tail musculature present in both the male and hermaphrodite as opposed to a male-specific one. The same head neurons were labeled by the pJM1 (*Punc-17small*:ChR2::YFP) construct in both sexes, and though the tails differ morphologically between the sexes, males and hermaphrodites share approximately the same neurological structure; there are only 87 neurons specific to the male [16]. If rgIs12 hermaphrodites displayed any tail curvature during or after exposure to a blue-light stimulus, then these behaviors would not be mating-specific. Two groups (n=30) of transgenic virgin day-1 hermaphrodites were selected at the sexually immature L4 stage and set up as described in the mating behavior assay.

The first group was exposed to a 10-second stimulus and filmed for the following three minutes, and the second group was exposed to an additional 10 seconds of low-intensity white background light. No worm in either group displayed any mating-like or idiosyncratic (coiling, self-seeking, or circling) behaviors when exposed to blue light or white light, thus suggesting that mating-like behaviors were specific to male worms (Figure 16).

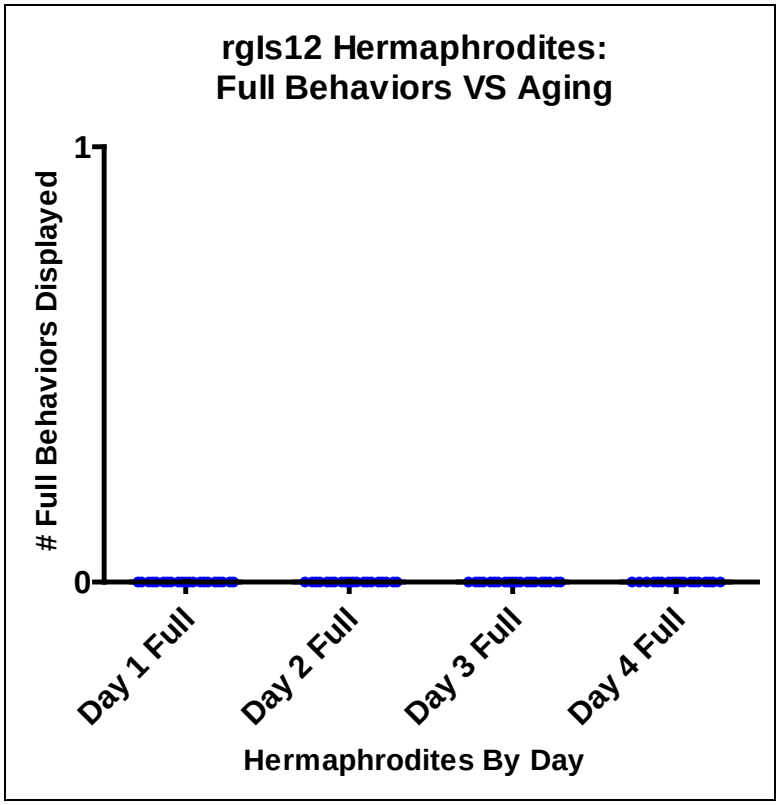

Figure 16. Mating-like behaviors displayed by rgIs12 hermaphrodites VS aging.
Each dot represents the number of mating-like behaviors displayed by a single rgIs12 hermaphrodite during the 190-second filming period consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a low-intensity white light background.

Mating-like behaviors are only displayed when cephalic cholinergic neurons are stimulated

The idiosyncratic circling, self-seeking, and whole body coiling behaviors are known to occur *in vivo* in wild-type male *C. elegans* worms. While spontaneous ventral tail curvature has not been observed *in vivo*, it was necessary to determine whether exposure to either high-intensity blue light or low-intensity white background light induced any of these behaviors in worms that had not been fed ATR. This would indicate what percentage, if any, of the mating-like behaviors observed could be

attributed to "noise" as the worm reacted in response to whole-body reaction to incident light. Two groups (n=30) of virgin transgenic male *C. elegans* worms were set up as described in the mating behavior assay procedure. However, the addition of ATR, the cofactor of Channelrhodopsin-2, was omitted. The experiment was carried out for four days. Each day after they reached sexual maturity, the first group of males was exposed to 10 seconds of high-intensity blue light stimulus, and filmed for the subsequent three minutes. Blue light stimulation was omitted for the second group, which was instead exposed to an additional 10 seconds of low-level white light. Two worms (1.7% response rate) in the blue-light stimulated group displayed one partial behavior on separate days. No full behaviors were displayed by any worm on any day in this group. No full or partial behaviors (0% response rate) were displayed on any day by the group of males exposed to white light (Figure 17).

Laser ablations of cholinergic neurons in the inner labial sensilla

Having found a subset of cholinergic neurons that, when stimulated, relayed a signal to the male tail, it was necessary to next elucidate the source of this signal. Ablation of individual cells and groups of cells with a laser microbeam has long been a tool used to determine the functions of cells in metabolic and behavioral pathways at all stages of the *C. elegans* life cycle [43]. Briefly, this technique uses a laser focused through the objective of a compound microscope to kill individual cells in the worm, which are

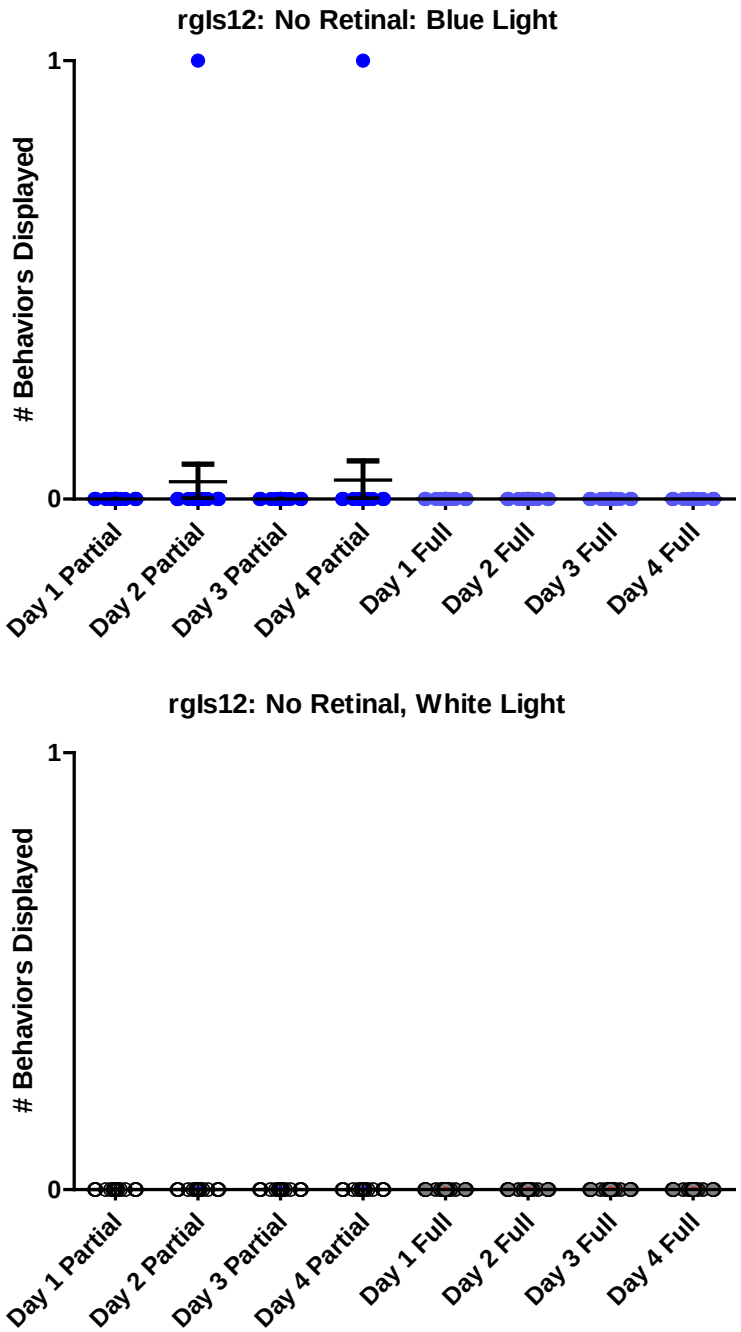

Figure 17. Mating-like behaviors displayed after exposing non-retinal-supplemented rgIs12 males to either high-intensity blue-light or low-intensity white-light stimulation.
(A) Mating-like behaviors displayed by rgIs12 males without retinal after exposure to high-intensity blue-light stimulus. Each dot represents the mating-like behavior response of a single rgIs12 male during a 190-second filming period consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a low-intensity white light background.
(B) No mating-like behaviors were displayed by rgIs12 males without retinal during a 190-second exposure to low-intensity white light.

visualized and focused using the microscope. The animal must be immobilized and the laser must be carefully fired in a small number of short pulses targeted to the nucleus, nucleolus, or cytoplasm of the target cell to minimalize refractory and thermal damage to neighboring cells and tissues [43]. The immobilization of the animal can cause behavioral defects, so care must be taken to (a) minimalize the amount of time the worm spends in surgery, and (b) expose a control "mock-ablated" group to the immobilization procedure but not to the laser.

The pJM1 (*Punc-17small*:ChR2::YFP) construct labeled 41 neurons in the head of the male worm (Figure 18). To simplify the search for a single neuron or group of neurons responsible for transmitting a cholinergic signal to the tail, it was decided to split the labeled neurons into three groups based on their anatomic location: (1) the group of neurons located posterior to the anterior bulb of the pharynx (IL2, IL2D, IL2V, URAD, URAV, and URB neuron pairs), (2) the group of neurons located anterior to the posterior bulb of the pharynx and posterior to the nerve ring (SMDD, SMDV, SAAV, RMD, RMDD, RMDV, SAAD, SIAD, SIAV, SIBD ,SIBV, SMBD, and SMBV neuron pairs), and (3) the group of neurons located ventral and ventral posterior to the posterior bulb of the pharynx (AIY and SABV neuron pairs, and the individual SABD neuron) (Figure 18A).

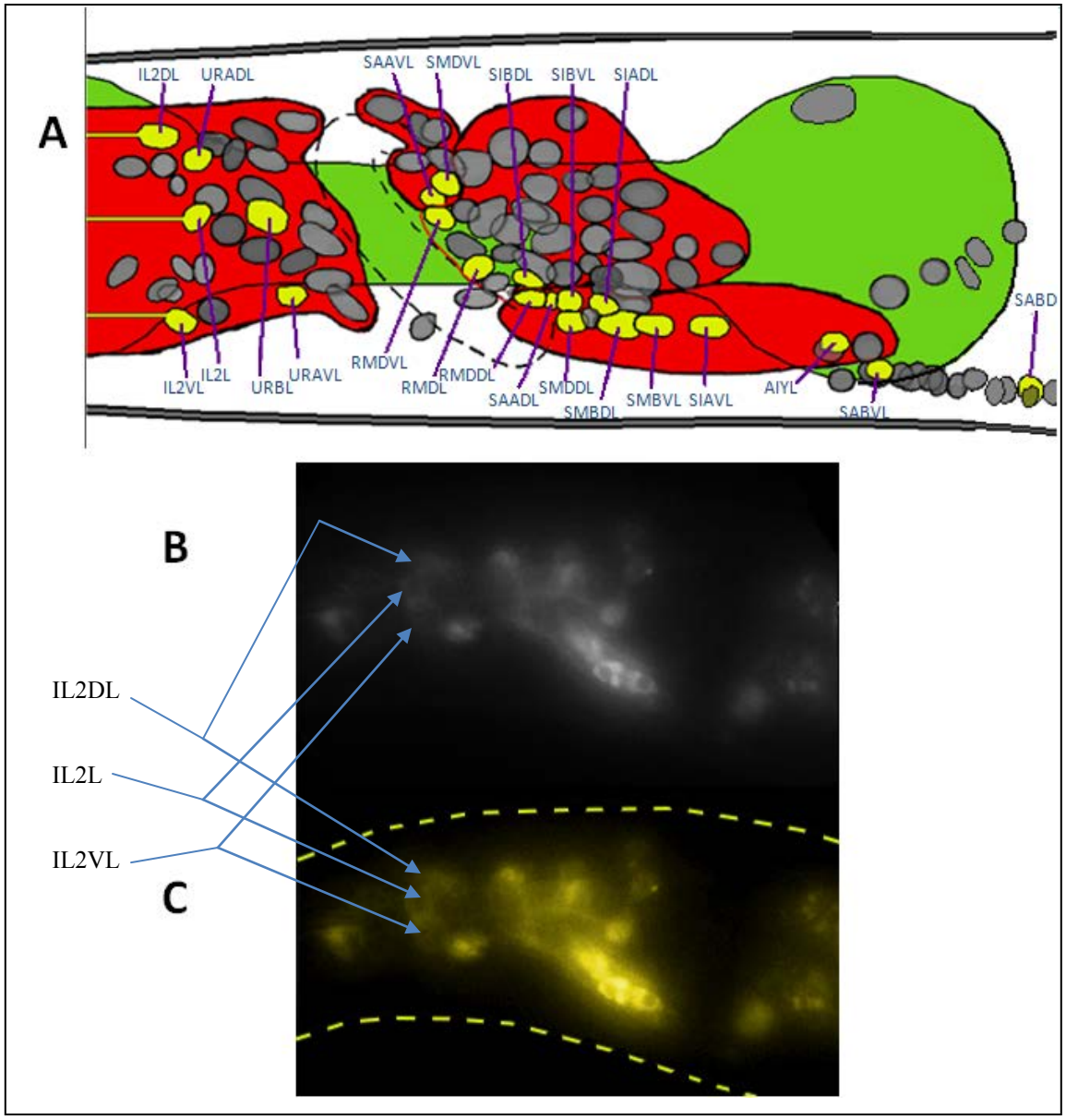

Figure 18. Anatomical distribution of cephalic cholinergic neurons labeled by pJM1 (*Punc-17small:ChR2::YFP)* in *C. elegans.*
(A) Cartoon of the cephalic cholinergic neurons, left lateral view, based on illustrations in [41]. 21 neurons are visible from the left side. All except the SABD neuron are paired with another neuron on the right side of the animal.
(B) Cephalic cholinergic neurons labeled by the PJM1 (*Punc-17small:ChR2::YFP*) construct, left lateral view. The three left IL2 neurons have been labeled.
(C) False-color image of cephalic cholinergic neurons in (B) to show YFP expression. An outline of the body wall has been added for anatomical perspective. The three left IL2 neurons have been labeled.

The third group of neurons (the AIY and SABV neuron pairs and the individual SABD neuron) were the most isolated and the easiest to visualize in the male *C. elegans* head. To gain familiarity with the technique of laser microbeam ablation, which requires great dexterity and attention to details to preserve the health of operated animals, these neurons were selected as the initial targets of laser ablation. Ablations were performed on L3-stage males using the procedure detailed in [43].

First, I analyzed the difference in mean displayed mating-like behaviors between mock-ablated (pad-immobilized and anaesthetic-exposed) day-1 virgin transgenic worms and the experimental group raised on NGM plates seeded with OP50 supplemented with 50uM ATR to determine whether exposure to the anaesthetic-soaked noble agar pad resulted in any difference in displayed behaviors. Sodium azide (NaN_3) is known to inhibit ATP synthesis in mitochondria by uncoupling proton transport through the F_0 subunit of ATP synthase [44]. There was no statistical difference in the number of full or partial mating-like behaviors displayed at any age between these groups (Wilcoxon Ranked-Sum test, $P<0.05$) (Figure 19).

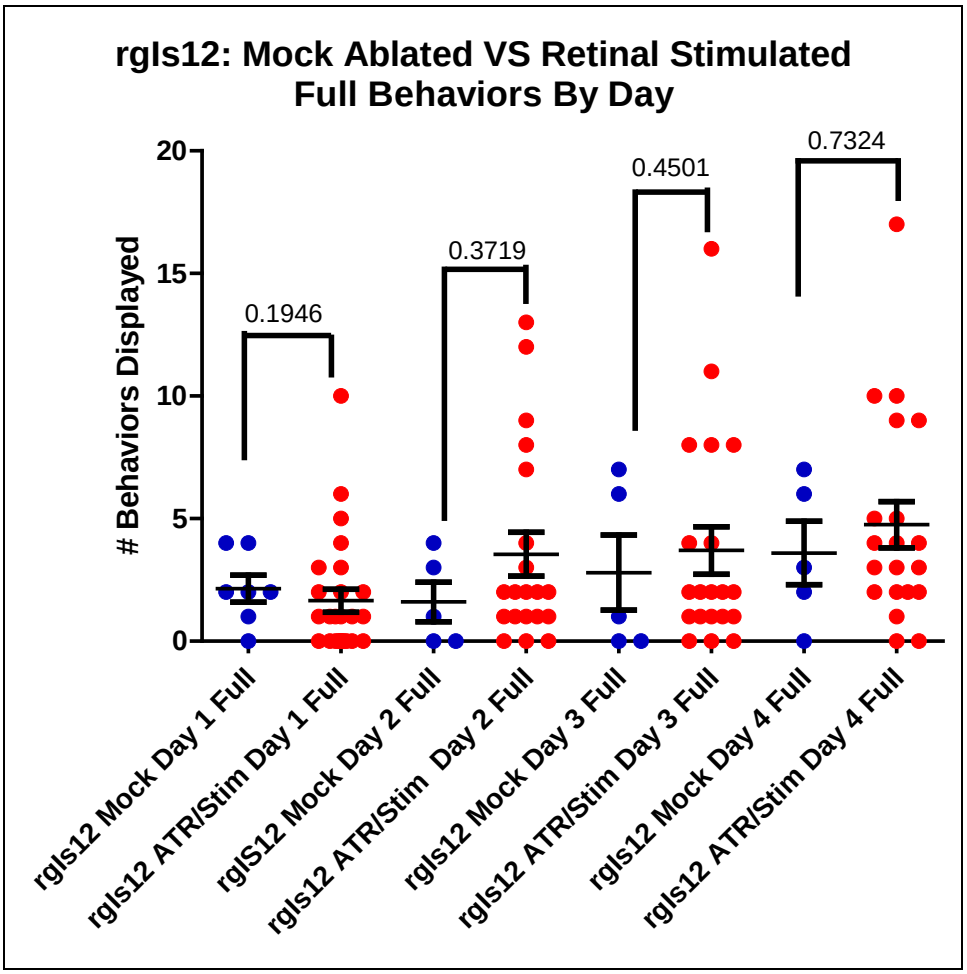

Figure 19. Comparison of mating-like behaviors displayed by mock-ablated rgIs12 males and unablated rgIs12 males. All worms were exposed to ATR for one hour filmed for a 190-second period consisting of a 10-second blue-light stimulation followed by a 180-second analysis period on a low-intensity white light background. Means were compared using the Wilcoxon Rank-Sum test.

Since mounting for surgery did not affect the mean mating-like behaviors displayed, I was confident that I could compare laser-ablated worms with mock-ablated worms and obtain reliable data. The group of neurons located posterior to the posterior `pharyngeal bulb was chosen as the first targets. The AIY neuron pair synapses with a number of non-cholinergic ciliated neurons in the amphid sensilla, some of which are

exposed directly to the external environment through cuticle pores, involved in thermotaxis and volatile and ionic chemotaxis [49,50]. The SABV neuron pair and the SABD neuron are cholinergic muscle neurons which extend axonal processes to innervate the dorsal and ventral quadrants of the head muscles [51-53]. Separate ablations of the AIY and SABV neuron pairs or the SABD individual neuron resulted in no significant difference in the mean number of full mating-like behaviors displayed between the ablated and mock-ablated groups (Figure 20).

The group of neurons located posterior to the anterior bulb of the pharynx (IL2, IL2D, IL2V, URAD, URAV, and URB neuron pairs) was chosen as the next set of targets due to their distinct separation from the cholinergic neurons near the posterior pharyngeal bulb. Additionally, the dendrites of the six IL2 neurons are known to be exposed directly to the external environment through pores in the cuticle, where they are involved in chemosensation (Figure 18) [54,55]. These neurons are known to regulate locomotion, certain motor responses, and other behaviors that require cholinergic transmission [56].

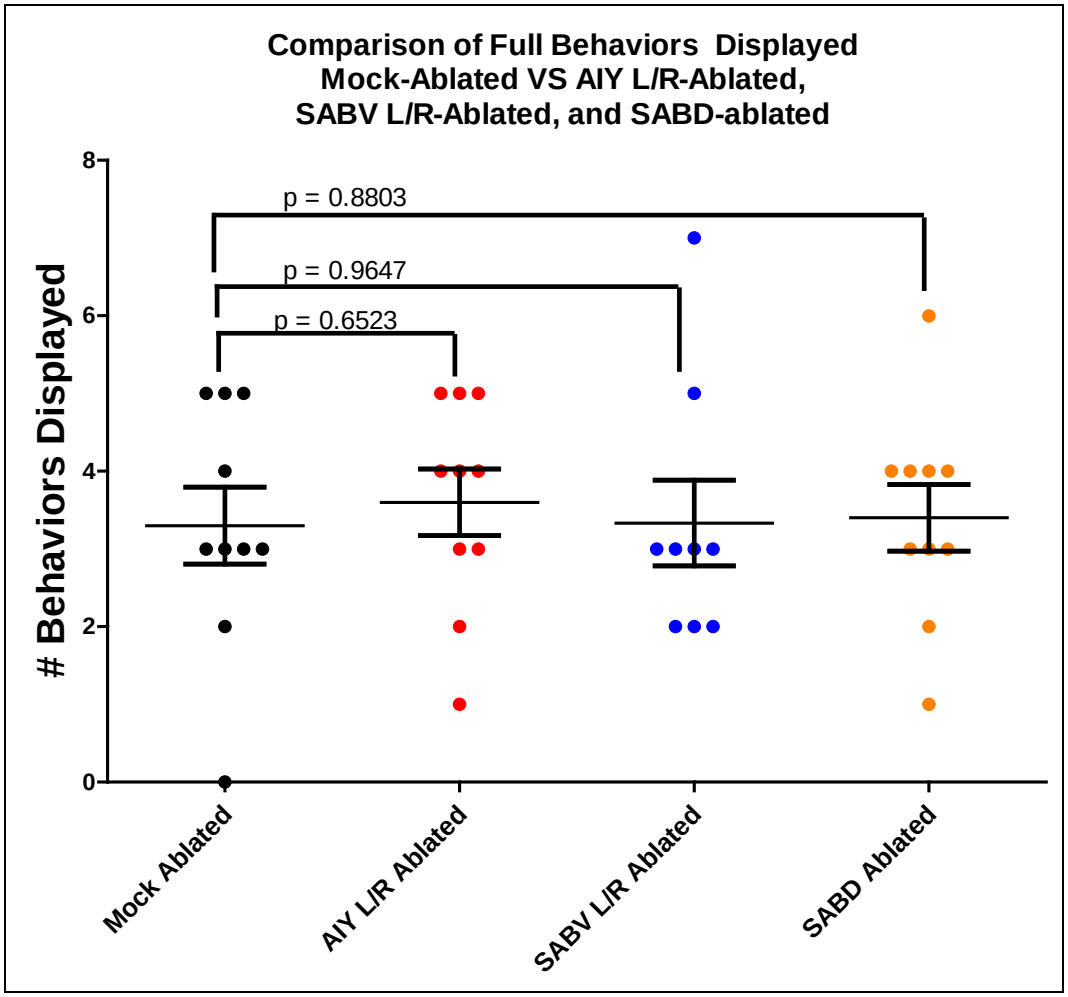

Figure 20. Comparison of mating-like behaviors displayed after blue-light stimulation in mock-ablated VS AIYL/R-ablated, SABVL/R-ablated, and SABD-ablated rgIs12 males.
Each dot represents the number of mating-like behaviors displayed by a male during a 190-second filming period consisting of 10 seconds of blue-light stimulation Means were compared using the Wilcoxon Rank-Sum test.

These six neurons form a ring immediately posterior of the anterior pharyngeal bulb and extend dendrites anteriorly along the length of the worm's head, each terminating in a raised structure in the lip of the cuticle, lined with a socket cell, through which they extend a cilium that directly sense the external environment (Figure 7) [55].

The six neurons forming the inner labial sensilla were ablated as described above. Each neuron pair was ablated first distally, then proximally to avoid excessive damage to surrounding tissues. Each ablated male was paired with a control "mock-ablated" male. Ablation of the IL2L/R neuron pair (n=10) resulted in a 60.7±25.7% reduction in displayed full mating-like behaviors between the ablated and mock-ablated groups which was statistically significant (Wilcoxon Ranked-Sum test, P=0.02) (Figure 21). Ablation of the IL2D neuron pair (n=10) resulted in a 45.5±24.3% difference in displayed full mating-like behaviors between ablated and mock-ablated groups which was not statistically significant (Wilcoxon Ranked-Sum test, P=0.092), although the variances were significantly different (Figure 22). Ablation of the IL2V neuron pair (n=10) resulted in a 84.1±35.3% difference in displayed full mating-like behavior between the ablated and mock-ablated groups which was statistically different (Wilcoxon Ranked-Sum test, P=0.0022) (Figure 23).

In all cases, ablation of IL2 neurons caused defects in the chemosensory ability of the worm, such that a large number of experimental males crawled aimlessly around the plate, often leaving the lawn of OP50 *E. coli* bacteria, crawling up the side of the plate, and desiccating. Even when plates were ringed with 100% glycerol, 14 of the 44 worms (31.8%) in which any pair of IL2 neurons was ablated crawled through the prohibitively viscous glycerol barrier and either died in the glycerol or crawled up the side of the plate and desiccated.

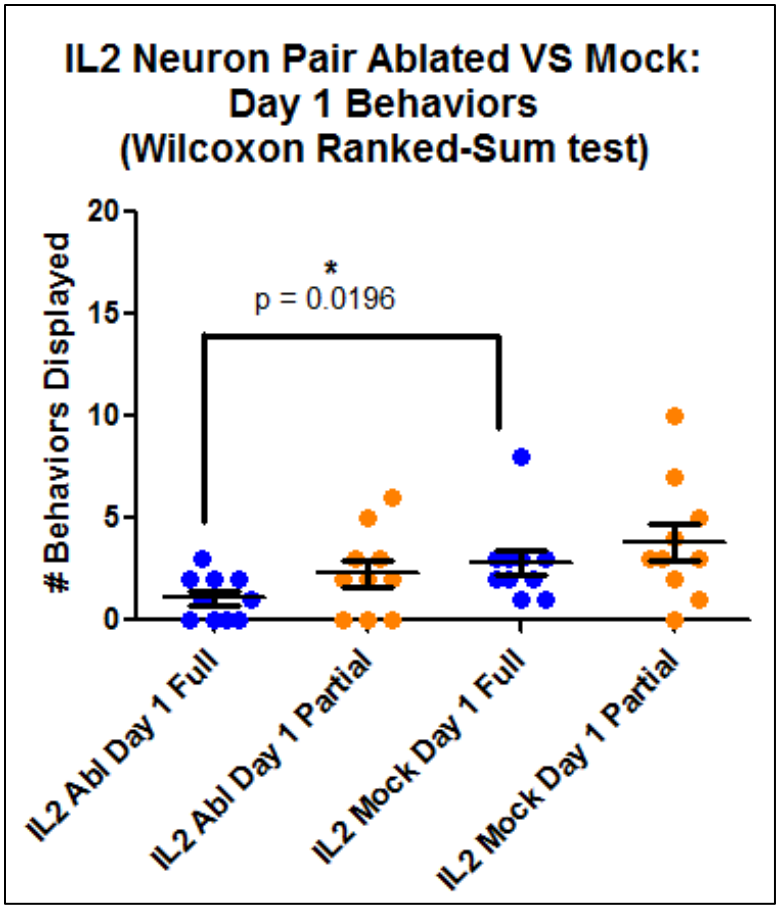

Figure 21. Mating-like behaviors displayed after blue-light stimulation in mock-ablated and IL2-ablated rgIs12 males. Each dot represents the number of mating-like behaviors displayed by a male during a 190-second filming period consisting of 10 seconds of blue-light stimulation Means were compared using the Wilcoxon Rank-Sum test.

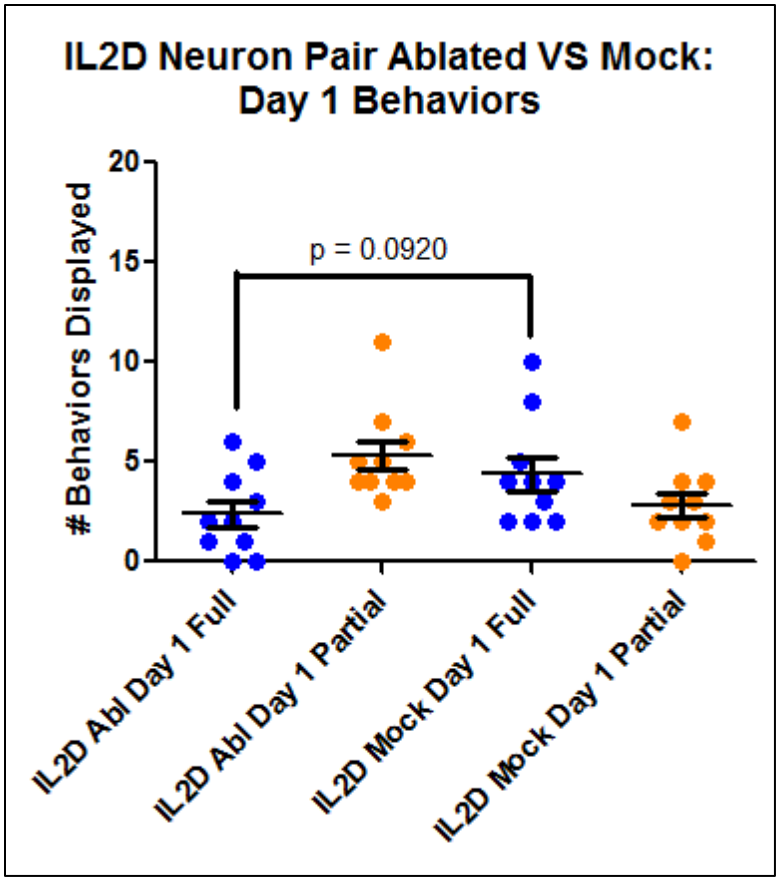

Figure 22. Mating-like behaviors displayed after blue-light stimulation in mock-ablated and IL2D-ablated rgIs12 males. Each dot represents the number of mating-like behaviors displayed by a male during a 190-second filming period consisting of 10 seconds of blue-light stimulation Means were compared using the Wilcoxon Rank-Sum test.

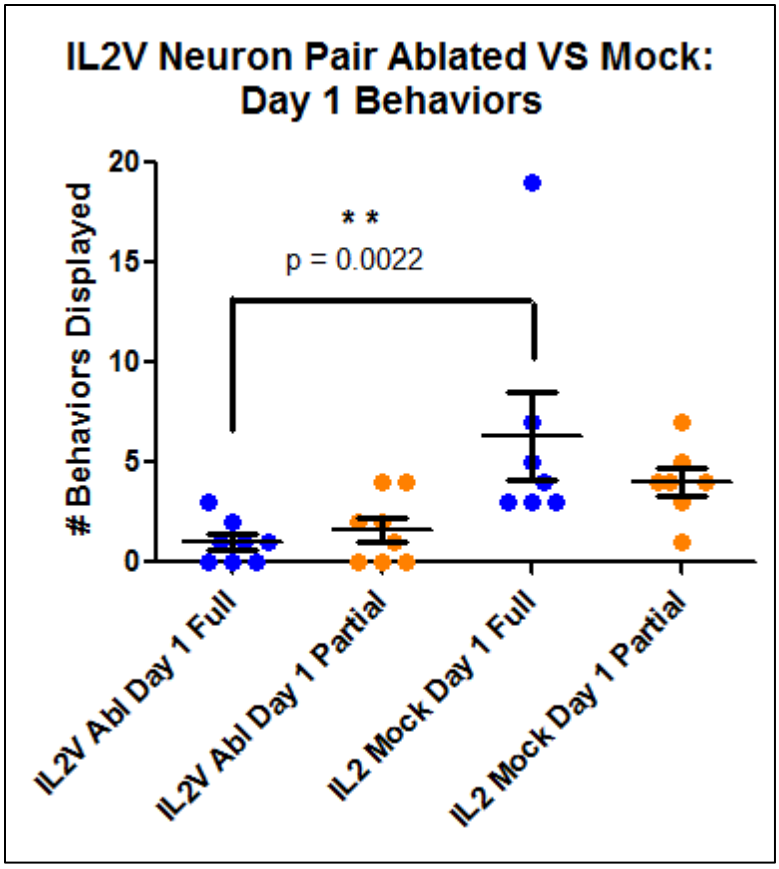

Figure 23. Mating-like behaviors displayed after blue-light stimulation in mock-ablated and IL2V-ablated rgIs12 males. Each dot represents the number of mating-like behaviors displayed by a male during a 190-second filming period consisting of 10 seconds of blue-light stimulation Means were compared using the Wilcoxon Rank-Sum test.

The effects of starvation upon displayed mating-like behaviors

It is known that the ability of a male *C. elegans* worm to successfully complete copulation and/or penetration of the hermaphrodite's vulva decays sharply after as little as one hour of starvation; previous studies have shown that such a short period of food deprivation increases the intermolecular interactions of the *unc-43*-encoded calcium/calmodulin-dependent (CAMKII) kinase with *egl-2*-encoded ether-a-go-go (EAG) K+ channels. CAMKII phosphorylates EAG K+ channels on their C-terminus. This phosphorylation, induced by a calcium influx triggered by starvation, increases the activity of the EAG K^+ channels, which hyperpolarizes the neurons of the mating circuit, lowering excitability of the cell [26,32].

Male worms lacking the *unc-103*-encoded ERG-like K+ channel spontaneously exhibited mating-like behaviors in the absence of a hermaphrodite, which was rescued when a short period of starvation increased the expression of EAG K+ channels in the sex muscles (dorsal and ventral protractors and anal depressor) [57,58]. Under food-deprived conditions, decreased excitability of the sex muscles suppresses the male mating circuit, leading to a change in motivation that permits the starving male to focus on foraging for food.

I therefore hypothesized that starvation of rgIs12 males on a bacteria-free substrate supplemented with ATR would induce the same hyperpolarization of the sex muscles, resulting in a decreased number of mating-like behaviors through possible CAMKII/EAG K+ channel interactions. Two groups of virgin day-1 transgenic male

worms were selected at the sexually immature L4 stage and incubated overnight on NGM plates seeded with OP50. Upon reaching the first day of adulthood, sexually mature worms from the first group (n=30) were transferred to NGM agar plates seeded with OP50 supplemented with 50uM ATR and incubated for 1 hour in the absence of light. The second group (n=33) was transferred by a mouth pipette with distilled water to a blank plate and allowed to crawl around for 1 minute to slough off bacteria adhered to the worm's cuticle. These males were then moved by a pipette with water to the center of a blank NGM agar plate ringed with 100% glycerol, onto which 20uL of 50uM ATR dissolved in distilled water was placed. These were also incubated for one hour in the absence of light.

The result of starvation on mating-like behaviors induced after ChR2-mediated direct stimulation of the cholinergic head neurons was contrary to the hypothesis stated above. Starved worms reacted robustly and almost immediately to the blue-light stimulus. They began crawling much more rapidly and displayed a 377.2% increase in displayed mating-like behaviors relative to the well-fed control group (Welch's t-test, $p = 0.0003$) (Figure 24). To elucidate whether this unexpected increase was a result of the combined stresses of starvation and noxious stimulus (the high-intensity blue light), a group of virgin transgenic males (n=31) was prepared as above, except that upon reaching adulthood, worms were transferred to individual blank glycerol-ringed NGM agar plates with a 20uL drop of distilled water in the center, and incubated for one hour in the absence of light. This group of males was stimulated as above. Starved worms raised without retinal displayed 44.7±24.5% fewer mating-like behaviors than the well-

fed group when exposed to high-intensity blue light. However, these two means were not quite significantly different according to Welch's t-test (P= (Welch's t-test, t(39)=1.82, P=0.0761) (Figure 24). A second group of males starved for eight hours on blank plates supplemented with 50uM ATR behaved similar to the 1-hour starved group relative to the well-fed group and the group starved without ATR (Figure 24).

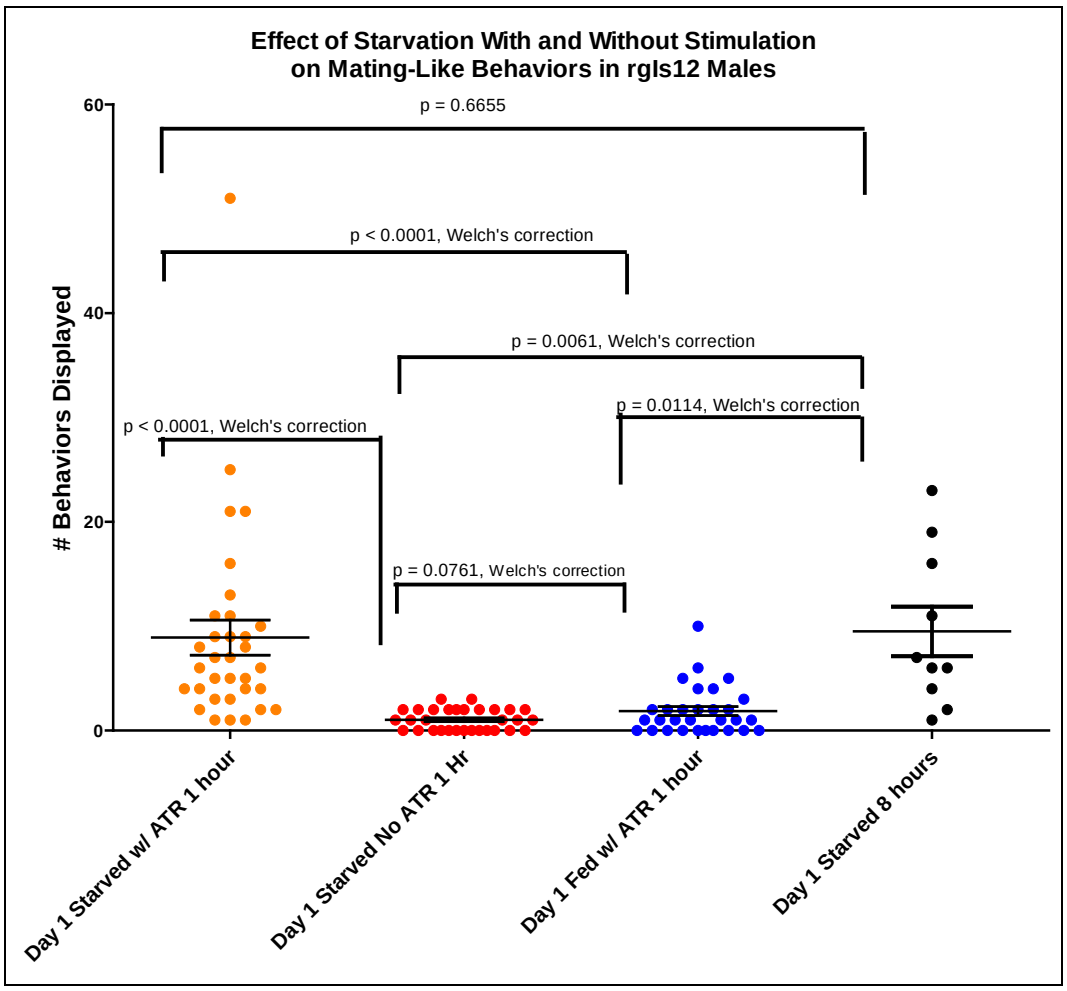

Figure 24. The effect of food-deprivation and/or stimulation on mating-like behaviors in rgIs12 male *C. elegans*.
Each dot represents the total number of full mating-like behaviors displayed by a single male during a 190-second filming period consisting of 10 seconds of blue-light stimulation followed by 180 seconds of analysis on a low-intensity white light background. Treatments were as indicated on the *x*-axis. Means were compared using the Wilcoxon Rank-Sum test.

Pharmacology of known AChR agonists with Starvation and/or Stimulation

The increase of displayed mating-like behaviors after starvation and stimulation provided useful data, but did not reveal anything about the state of the mating system in either fed or starved worms that were *not* stimulated. It has been shown that starvation suppresses spicule protraction, the final output of the mating circuit, and therefore potency, but the mating behaviors induced after ChR2-mediated stimulation of the cholinergic head neurons generally did not include spicule protraction, which occurred in 1 of 17 rgEx551 (5.8%) males and 3 of 60 rgIs12 (5.0%) males. I decided to use pharmacology assays to determine whether starvation in the absence of cues from a hermaphrodite actually increased the *propensity* of the mating circuit in the tail to fire upon reception of induced cholinergic signaling from the head, or whether starvation simply increased the ability of those signals to generate a partial subset of the processive behaviors involved in male copulation.

Drug tests were performed using the method of [31]. Spicule protraction was used as a metric of stimulation of the mating circuit by the treatment applied (fed with ATR and stimulation, fed without ATR or stimulation, starved with ATR and stimulation, and starved without ATR or stimulation). These drugs would also indicate which subtypes of acetylcholine receptors were involved in mediating signaling from the cholinergic head neurons to the mating circuit in the tail. Nicotine solely activates the ACR-16 subunit of the levamisole-independent ionotropic acetylcholine receptor [59]. Levamisole acts through the UNC-38 and UNC-63 alpha subunits and the non-alpha

UNC-29 subunit of the ionotropic levamisole-dependent acetylcholine receptor (L-AChR), as well as the UNC-68 ryanodine receptor in the sex muscles [60], which mediates spicule protraction via cholinergic signaling from the PCB and PCC neurons in the postcloacal sensilla [28,31]. Arecoline is an indiscriminate ACh agonist that binds to a wide-range of ionotropic and G-protein-coupled acetylcholine receptors.

The effect of the drugs on spicule protraction rate was measured by calculating an approximate EC_{50} for each group of rgIs12 males. An EC_{50} is a theoretical measurement of the concentration of acetylcholine agonist that would cause 50% of the rgIs12 males in a group (n=30) to protract their copulatory spicules. A smaller EC_{50} indicated a higher sensitivity to the drug in question.

In arecoline, fed and stimulated rgIs12 males had the lowest EC_{50}, followed by starved stimulated males, then fed unstimulated males, and finally starved unstimulated males (Figure 25A). In nicotine, fed stimulated males had the lowest EC_{50}. Both starved stimulated and starved unstimulated males had a lower EC_{50} than fed unstimulated males (Figure 25B). In levamisole, fed unstimulated males had the lowest EC_{50}, followed by starved unstimulated males and fed stimulated males. Starved stimulated males were highly resistant to protracting their spicules even at high concentrations of levamisole (Figure 25C).

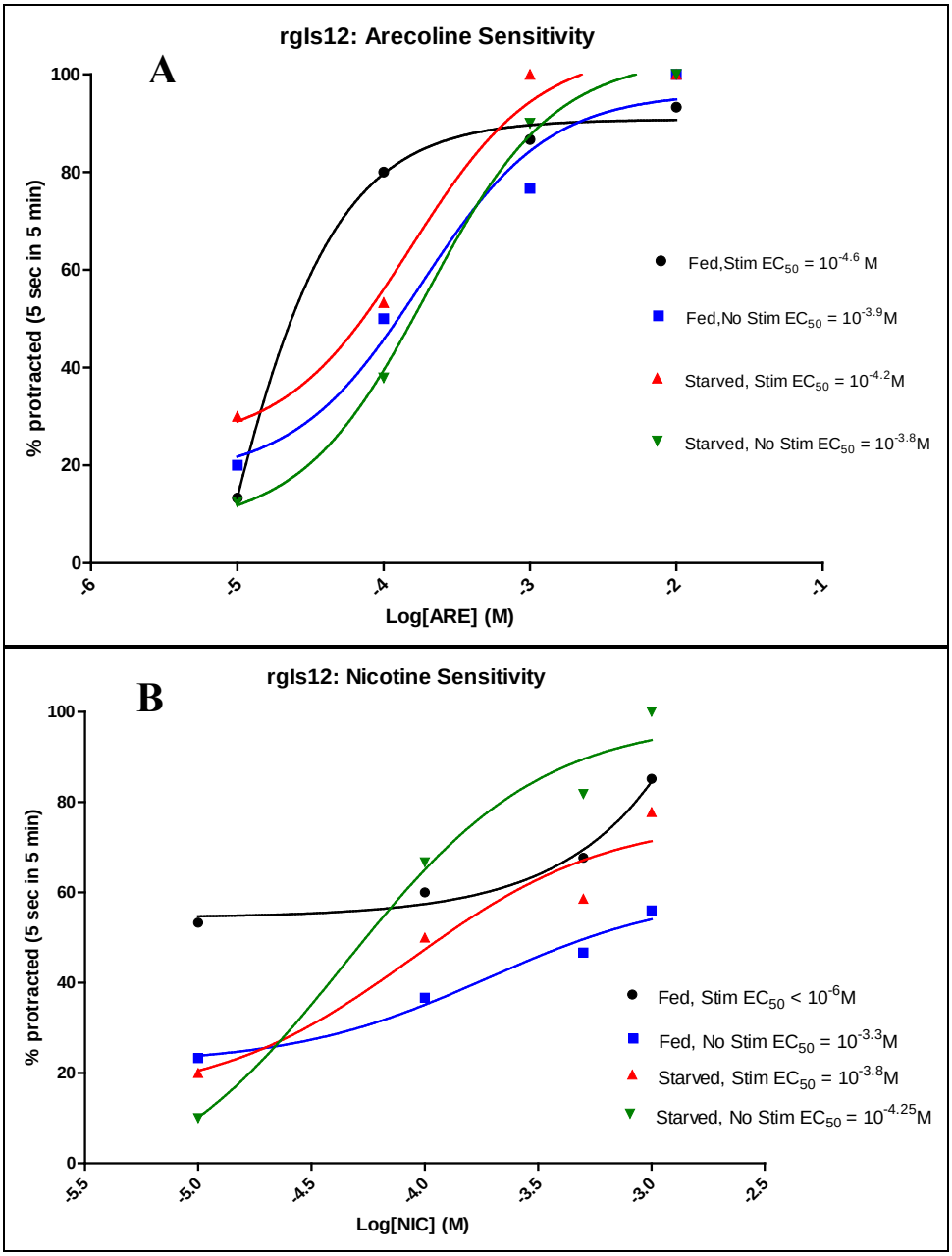

Figure 25. Sensitivity of rgIs12 strain to various acetylcholine receptor agonists, as measured by % spicule protraction.
Each dot represents the % of males in a group (n=30) that protracted their spicules in response to exposure to the concentration of acetylcholine agonist indicated on the *x*-axis (n=30). EC_{50} is defined as the concentration of acetylcholine receptor agonist that caused 50% of males (n=30) to protract their copulatory spicules.
(A) Dose-response curves of rgIs12 males to the indiscriminate acetylcholine receptor agonist arecoline (ARE) under fed and starved conditions, with or without stimulation.
(B) Dose-response curves of rgIs12 males to the nicotine-dependent ionotropic acetylcholine receptor agonist nicotine (NIC) under fed and starved conditions, with or without stimulation.
(C) Dose-response curves of rgIs12 males to the levamisole-dependent ionotropic acetylcholine receptor agonist levamisole (LEV) under fed and starved conditions, with or without stimulation.

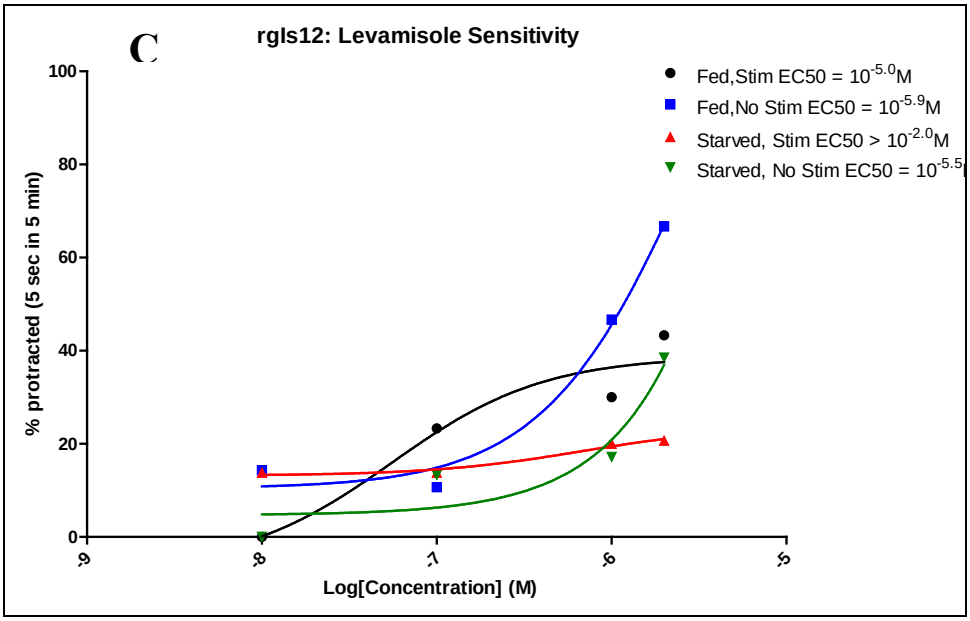

Figure 25 Continued.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary of experiments

The purpose of this study was to determine what role, if any, the cephalic cholinergic neurons played in regulating any mating-like behaviors in the tail of the male *C. elegans* worm. Previous studies have elucidated the connectivity and regulation of the sex-specific male mating apparatus, but to date no work has been carried out to determine if non-sex-specific structures modulate the mating execution circuits in the male. I used ChR2-mediated inducible activation of the entire set of cephalic cholinergic neurons to measure resulting mating-like behavioral effects. I found that a specific subset of cephalic cholinergic neurons, the IL2 and IL2V neuron pairs, generate and relay a signal to the tail of the worm that increases the propensity of the mating apparatus to precociously perform mating-like behaviors in the absence of a hermaphrodite or its mating cues. I showed that direct stimulation of this circuit under both fed and food-deprived conditions increased the likelihood that a worm would display these behaviors.

Stimulation of cephalic cholinergic neurons induces male-specific mating-like behaviors in the tail

By specifically stimulating the cephalic cholinergic neurons using ChR2, I was able to rapidly induce a stereotyped male-specific mating-like ventral curvature of the male's tail. I proved that these behaviors were specific to male *C. elegans*. No rgIs12 hermaphrodite ever displayed any degree of mating-like ventral tail curvature in response to blue-light stimulus, even though the pJM1 (Punc-17small:ChR2::YFP) construct labeled the same set of 41 neurons in both sexes (Figure 15, 17). I conclude that the mating-like behavioral response in the tail to stimulation of the cephalic cholinergic neurons represents a novel connectivity and function of these neurons that is unique to the male.

Mating-like behavioral display did not occur frequently *in vivo* or in response to a blue light stimulus in worms that were not supplemented with ATR. This indicates that the behaviors I saw were not simply a response by the worm to the stress of being exposed to high-intensity illumination, a noxious and dangerous condition that can kill the worm [10].

The mating-like behaviors increased in frequency during filming as males aged, mimicking the decreased excitability of the male mating and spicule protraction circuits seen in aging worms [37]. Aging causes the neurons and muscles involved in the mating circuit to hypo-polarize, so that the circuit as a whole is much easier to fire as an animal ages. Hypo-polarization of the components of the mating circuit translates into

prematurely-initiated spicule prodding and spicule protraction behaviors that occur before the male has achieved proper location at the vulva, resulting in a decreased ability to perform the complicated mating behavior to completion. *C. elegans* males thus experience a decline in mating potency that correlates with age. This evidence lends credence to my conclusion that ventral tail-curling behaviors occurring as a result of direct stimulation of cephalic cholinergic neurons are mating-specific.

The IL2 and IL2V neuron pairs are involved in regulating the male mating circuit's response to stimulation of cephalic cholinergic neurons

Paired ablations of the cephalic cholinergic neurons identified the IL2L/R and the IL2VL/R sensory neuron pairs, located in the inner labial sensilla, resulted in statistically significant decreases in the number of mating-like behaviors displayed during identical filming periods relative to control mock-ablated worms. The ablation of the IL2D neuron pair resulted in a weakly-insignificant change in the mean number of mating-like behaviors displayed (Wilcoxon Ranked-Sum test, P=0.07). The stress of laser microsurgery alone was not responsible for the decreases in mating-like behaviors. I can therefore conclude that the IL2L, IL2R, IL2VL, and IL2VR neurons are as at least partially responsible for generating and relaying the signal that mediated mating-specific tail curvature in the absence of a hermaphrodite. These neurons are suitable candidates as receptors of hermaphrodite cues because they extend ciliated processes through the cuticle of the male that directly sense the external environment, and because they are

known to be involved in chemosensation and the regulation of motor responses such as nictation and photophobia to environmental stimuli [10,54-56]. Additionally, chemoattraction to hermaphrodite mating cues is modulated by TRPV (transient receptor potential vanilloid) receptors in the male's AWA, AWC, and CEM neurons encoded by the osm-9, ocr-1, and ocr-2 genes [61].

It has been proven that hermaphrodite *C. elegans* worms emit a family of small diffusible ascaroside chemoattractant molecules (which contain ascarylose) that alter the motivation state of male worms, causing them to crawl towards the source of the signal [22,45]. The fact that sensation of the hermaphrodite's chemical cues mediates a long-lasting behavioral change in the male indicates that the neural circuit controlling this behavior retains a memory of stimulation by a hermaphrodite cue. Based on this evidence, there are two possible explanations for the role that the neurons of the inner labial sensilla play in regulating mating behavior. First, I propose that these neurons may sense the presence or lack of food in the environment, and consequently up-regulate or down-regulate mating motivation, respectively. In this scenario, the IL2 neurons form the circuit that receives environmental information about food levels and relays the existence of favorable nutrient conditions to the mating apparatus in the male's tail. I propose that this signal increases the excitability of the mating circuit, which is normally downregulated when mates are absent.

Second, and less likely, I propose that the IL2 and IL2V neuron pairs, along with other potential cephalic cholinergic candidates, are an additional part of the circuit that receives ascaroside hermaphrodite mating cues from the environment and relays the

existence of positive mating conditions to the mating apparatus in the tail. Previous studies have already identified the molecules that cause this attraction, and have shown that the AWA, AWC, and CEM neurons receive these signals through TRPV receptors in these neurons [45,61]. It is therefore likely that, if the IL2 neurons are involved in sensing mating cues, they sense a unidentified signaling molecule different from the ascaroside class of glycolipids. An objection to this conclusion could point out that when males sense a hermaphrodite and begin crawling toward it, they do not display precocious mating-like ventral tail curvature. I counter this hypothetic assertion by interpreting the degree of stimulus received by the chemosensory cephalic cholinergic neurons. The amount of stimulus caused by reception of a hermaphrodite's mating cues is likely not as large as the amount of stimulus that these neurons received during artificial direct ChR-2 mediated stimulation. I propose that low-level stimulation (from a hermaphrodite cue) increases the excitability of sex muscles and their related neurons (effectively "priming" the mating circuit), while a much larger stimulus (either blue-light or white-light) very quickly induces mating-like behaviors in the tail by hyper-exciting the mating circuit with exogenous signal.

The circuit mediating cephalic cholinergic regulation of mating behavior retains a memory of stimulation

The onset of mating-like behavior in the tail occurred after a statistically significant latency from the cessation of stimulation of cephalic cholinergic neurons. For

most worms analyzed, partial and full mating-like behaviors were not displayed until after the 10-second stimulation period. The mean latency of full mating-like behaviors, did not significantly differ with age. The amount of stimulus necessary to cause cephalic cholinergic neurons to generate a signal did not change. Since the mating circuit hypo-polarizes with age, the evidence indicates that the cephalic cholinergic neurons involved in generating and relaying a signal to the mating structures in the tail do *not* hypo-polarize with age. At any point in time during the animal's lifetime, they are capable of generating this signal as a response to the same amount of stimulus. The mean latency of partial behaviors decreased significantly as the worm aged (Figure 14). Since the cephalic cholinergic neurons do not appear to hypo-polarize with age, but the components of the mating circuit do, this result indicates that components of the *mating circuit* required less and less stimulus to fire as the animal aged. I can therefore conclude that the circuit mediating cephalic cholinergic regulation of mating behavior retains a memory of the stimulus long after it is removed, and modulates a lasting state change in the excitability of the components of the male mating circuit. The cephalic cholinergic circuit does not experience a change in excitability as the animal ages.

Under starvation conditions, which normally decrease the excitability of the sex muscles, the number of tail-curling behaviors declined. However, specific stimulation of the cephalic cholinergic neurons was able to rescue the decline in mating-like behavior display, and caused an even greater output of tail-curling behaviors than in fed males. Starvation induces Ca^{2+} transients that activate *unc-103*-encoded ERG-like K+ channels, which increase the likelihood that *unc-43*-encoded CAMKII phosphorylates *egl-2*-

encoded ether-a-go-go (EAG) K+ channels. Starvation therefore results in the hyperpolarization of the neurons and muscles of the mating circuit, effectively shutting down the capacity and motivation for mating until the animal locates food [31].

If a subset of the cephalic cholinergic neurons is responsible for sensing hermaphrodite mating cues, then these neurons could initiate an override of the desire to eat. It has been shown that male worms that receive hermaphrodite mating cues will leave the security of a lawn of *E. coli* to search for a mate [22]. This explains why direct stimulation of starved worms increased the number of mating-like behaviors displayed. Worms that were starved but not stimulated, however, displayed a mean number of mating-like behaviors that was not statistically different from that of fed and stimulated worms. I propose that starvation puts the male into a generalized "fight-or-flight" mode, during which the extreme motivation to find food can upregulate the excitability of food-sensing circuits. Since the IL2 neurons are involved in sensing food, they would be upregulated in this scenario. They would therefore be so sensitive to stimulation during starvation that the photophobic stress induced by high-intensity blue light provides sufficient stimulus to cross the excitatory threshold of these neurons, causing them to fire directly or as a result of indirect stimulation of other neurons. I can therefore conclude that a subset of the cephalic cholinergic neurons have a regulatory role on the excitability state of the male mating system in the tail.

Possible mechanisms of communication between the cephalic cholinergic neurons and the mating circuit in the tail

Ventral tail curvature is an important step in male mating that permits close contact with the hermaphrodite and proper positioning of the copulatory spicules over the vulva. Regulation of ventral tail curvature is carried out by inputs from multiple male-specific sensory structures. It has been shown that, under food-limited conditions, the NSM neurons in the male's head sense the decrease in pharyngeal pumping rate and attenuate sex muscle excitability [25]. Conversely, when the ray neurons sense the physical presence of the hermaphrodite, attenuate the activity of pharyngeal pumping [25]. Combined serotonergic signaling from the RnB ray neurons and the male-specific CP motor-neurons are required to initiate and maintain proper ventral tail-curling so that the male can press his mating structures firmly against the hermaphrodite [11]. Physical sensation of the vulva is mediated by the male's PCS and hook sensilla. Signaling from these neurons to the sex muscles (specifically the oblique muscles) mediate ventral tail curvature in order to position the mating apparati prior to spicule prodding (Figure 3) [29-32].

I propose that the IL2 and IL2V neuron pairs, possibly in concert with other cephalic cholinergic head neurons, either sense the presence of food in the environment or, less likely, sense the hermaphrodite-emitted diffusible mating cues identified in [22] and [45], either before or during physical contact with the hermaphrodite. I further propose five mechanisms by which the cephalic cholinergic neurons generate and relay a

signal to the mating circuit in the tail. First, reception of either food cues or an unidentified class of hermaphrodite signaling compound by the IL2 neurons can signal directly or humorally to the NSM amphid neurons. During conditions of starvation, my data has shown that mating-like behaviors in the tail increase upon stimulation of the cephalic cholinergic neurons. Therefore, IL2-mediated signaling may inhibit the NSM neurons' inhibition of tropomyosin in the tail. During periods of satiation, sensation of a hermaphrodite by the IL2 neurons may attenuate the NSM neurons' attenuationsex muscle excitation. Second, the sensation of hermaphrodite cues by the IL2 neurons may act in concert with serotonergic signaling from the rays and CP motor-neurons to regulate ventral tail-curling upon initial contact with the hermaphrodite. There is no known connectivity between the IL2 neurons and either the rays or the CP motor-neurons [15]. I propose that this signal is either communicated indirectly to the tail via the NSM neurons, or propagated humorally from the cephalic cholinergic neurons. Given that my data showed a latency between stimulation of the cephalic cholinergic neurons and inception of mating-like behavior display in the tail, the latter theory of a diffusible humoral signal, which takes a longer time to travel to the mating structures in the tail, is more strongly suggested. Third, I propose that signaling from cephalic cholinergic neurons acts in tandem with the physical sensation of the hermaphrodite's vulva by the hook and PCS neurons to cause contractions of the sex muscles, specifically the oblique muscles. As there is no known physical or chemical connection between the IL2 neurons and either the HOA and HOB neuron pairs in the hook sensillum or the PCA, PCB, and PCC neuron pairs in the PCS, and my data showed a latency between

stimulation of the cephalic cholinergic neurons and mating-like activity in the tail, I propose that this type of signaling is mediated humorally [15]. Fourth, I propose that excitability of the sex muscles (specifically the oblique muscles) is partially regulated by signaling from the IL2 neurons. Detection of hermaphrodite cues by the IL2 neurons may mediate signaling to the sex muscles, which increases their excitability and renders them more prone to fire, inducing ventral tail-curling in response to physical sensation of the hermaphrodite's cuticle or vulva. There is no known connectivity between the cephalic cholinergic neurons and any of the sex muscles [15]. Fifth, and least likely, I propose that there may be an as-yet undetected physical or chemical junction between the IL2 neurons and any of the neurons or muscles involved in ventral tail curvature. This hypothetical connection can be either directly between the IL2 neurons and the mating apparatus (which would require an axon spanning almost the entire length of the adult male worm) or indirectly through one or multiple interneurons.

Stimulation of cephalic cholinergic neurons increases the sensitivity of rgIs12 males to known AChR agonists

The sensitivity of a male *C. elegans* worm to these ACh agonists has been used as a proxy in previous studies to measure excitability of the spicule protraction circuit; in conditions where mating motivation is increased, the cholinergic neurons in the spicule protraction circuit will require less of a given ACh agonist to fire, meaning that the spicules will be easier to protract [25]. The pharmacology assays performed indicate the

likelihood that an rgIs12 male will protract his copulatory spicules when exposed to increasing concentrations of acetylcholine receptor agonists. I have proposed that the cephalic cholinergic neurons are involved in sensing food or a mate in the environment, relaying that information to the tail, and regulating the excitability of the male's sex muscles. The response pattern of rgIs12 males to arecoline, an indiscriminate acetylcholine receptor agonist that acts on many families of ionotropic (nicotinic) and G-protein-coupled (muscarinic) acetylcholine receptors, follows the pattern that would be expected if the cephalic cholinergic neurons regulate sex muscle excitability based on external environmental cues. Starvation decreased the propensity of rgIs12 males to protract their copulatory spicules in the presence of increasing doses of arecoline, while stimulation both increased the chance that fed males would protract their spicules, and partially rescued the decrease in spicule protraction associated with starvation (Figure 25 A-C). However, the response of rgIs12 males to the specific nicotine-dependent ionotropic acetylcholine receptor agonist nicotine and the specific levamisole-dependent acetylcholine receptor agonist levamisole conflicts with this proposed model. Arecoline acts on most of the acetylcholine receptors within the worm, while these two agonists only act on small subsets of them. Therefore, while the response of rgIs12 males to arecoline supports my hypothesis that cephalic cholinergic neurons sense favorable or unfavorable environmental conditions and send signals that alter the excitability of the sex muscles, the data from nicotine and levamisole suggest that the mechanism of this regulation is more complex than a simple "on-off" switch, and merits further study.

Future experiments

A number of experiments can be performed to elucidate the type and method of signaling from the IL2 neurons. I can analyze which specific sex muscles, if any, in the tail, are activated as a result of stimulation of the IL2 neurons. The GCaMP protein is an engineered calcium indicator comprised of a fusion of calmodulin (CaM), GFP, and the M13 domain of the myosin light chain kinase [62]. When calcium is present, it binds to CaM, which undergoes a conformational change and binds to M13, activating GFP fluorescence. Calcium concentrations increase in the sex muscles when they are contracting. GCaMP has been used to show specific muscular activity during *C. elegans* male mating [25,26,32,37]. I can express GCaMP under the control of various sex-muscle-specific promoters to ascertain which muscles play an active role in ventral tail-curvature in response to ChR2-mediated stimulation of cephalic cholinergic neurons.

The IL2 neurons can be analyzed using ChR2-mediated stimulation to determine whether they release acetylcholine in response to stimulation. The *klp-6* promoter expresses specifically in the IL2 neurons [56]. The KLP-6 protein is also essential for male mating [27]. Cholinergic signaling can be interrupted by a loss-of-function mutation in either EGL-8 (PLCβ – the beta subunit of phospholipase C , which hydrolyzes PIP_2 to DAG and IP_3) or EGL-30 (the alpha subunit of the G_q protein which phosphorylates phospholipase C). Such a mutation inhibits DAG production, preventing the binding of DAG to UNC-13 (DAG binding protein) and shutting down synaptic transmission [63]. A transgenic strain of *C. elegans* could be created bearing the pJM1

(*Punc-17small:ChR2::*YFP) construct and the *klp-6* promoter driving either egl-8(dominant negative) or egl-30(dominant negative), which would express specifically in the IL2 neurons. The worm could then be stimulated using the methods above. If mating-like behaviors in the tail are reduced or abolished, this would indicate that the signal originating from the IL2 neurons is cholinergic.

Currently, the rgIs12 line expresses ChR2 in the entire subset of cephalic cholinergic neurons. While the PJM1 (Punc-17small:ChR2::YFP) construct could be expressed under the control of the IL2-specific klp-6 promoter [56] to determine the effect that stimulation of solely these neurons has on mating-like behaviors in the tail, it would not provide information on the involvement of any of the other cephalic cholinergic neurons. A simpler experiment can be conducted to confirm or rule out involvement of any of these other neurons. Using an Olympus IX81 compound microscope with a Uniblitz model VCM-D1 shutter driver controlled by the HCImage program, I can selectively stimulate hand-drawn regions of interest (ROI's) in the head of a male worm. This specialized microscope uses an array of adjustable mirrors to mask all but the selected ROI from light exposure. I can use this scope to highlight the three groups of cephalic cholinergic neurons that I discussed above, and perform a mating behavior assay immediately after stimulation to determine if neurons in any of these regions contribute to the display of mating-like behaviors in the tail.

I can also confirm or rule out whether the IL2 neurons mediate ventral tail-curvature through the ray neurons. I can ablate the ray neurons in groups or in their entirety as in [14] using the procedure of (Figure 3) [43]. I can then perform a

stimulation and mating behavior assay as described above. If mating-like behavior display declines or is abolished in the absence of any subset of the ray neurons, then this would indicate that signaling from the cephalic cholinergic neurons mediates ventral tail curvature through the ray neurons and their serotonergic signaling to the sex muscles.

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